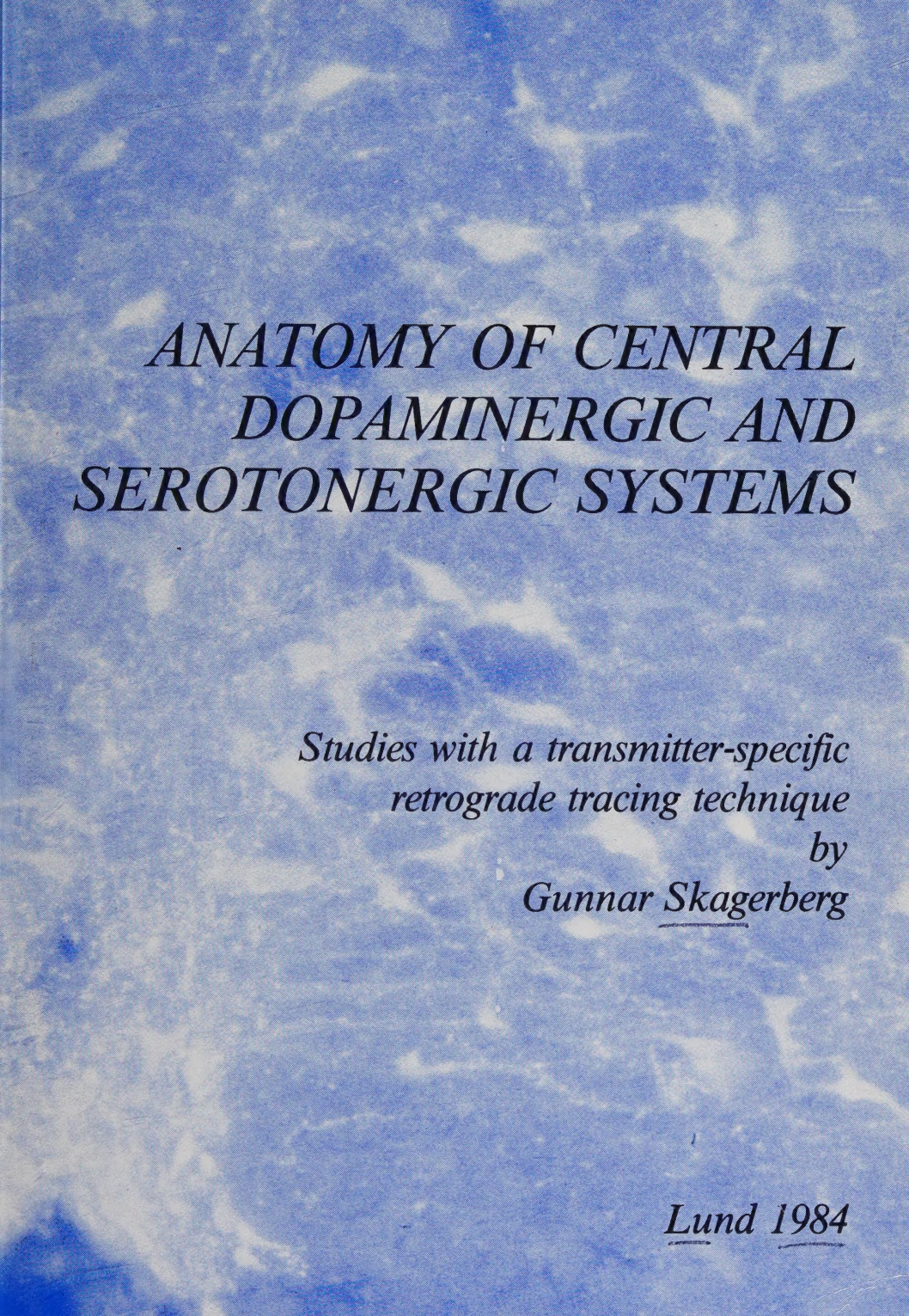




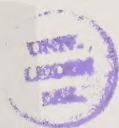
ANATOMY OF CENTRAL DOPAMINERGIC AND SEROTONERGIC SYSTEMS

*Studies with a transmitter-specific
retrograde tracing technique*

by

Gunnar Skagerberg

Lund 1984



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AND SEROTONERGIC SYSTEMS**

**Studies with a transmitter specific
retrograde tracing technique**

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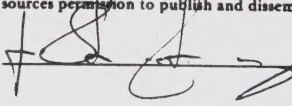
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Abstract This thesis is concerned with the development and application of anatomical methods suitable for studying central monoaminergic (MA) systems. 1) By taking advantage of neurotoxin regimens that produced a selective removal of central noradrenergic (NA) systems it was possible to use the aldehyde-induced fluorescence method for mapping the distribution of dopamine (DA) fibres also in areas where NA is the predominating catecholamine. Several previously unknown areas of DA-innervation were revealed, most notably in the (a) dorsal and intermediate parts of the spinal cord and in (b) the supraoptic and paraventricular nuclei in the hypothalamus. 2) The fluorescent retrograde tracer True Blue was found to be well suited for use in combination with aldehyde-induced MA-fluorescence for tracing the origins of monoaminergic projections. (a) The spinal DA innervation was found to originate exclusively in the diencephalic A11 group in which the spinal projecting DA neurons all project to and through the same areas of the cord. In contrast, (b) the spinal serotonin (5HT) system displayed a high degree of topographical order with a strict segregation of 5HT neurons located in the pons and medulla and projecting to dorsal, intermediate or ventral regions of the spinal cord. (c) Both in the diencephalon and in the caudal brainstem many spinal-projecting non-MA cells were found intermingled with the spinal-projecting DA and 5-HT cells. d) The DA innervation of the lateral habenula was found to be confined to the medial and caudal part of this nucleus. This DA innervation was found to originate, together with a quantitatively larger non-DA projection, in the ventral tegmental area and to reach the habenula in association with the fasciculus retroflexus.			
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The general idea is always an abstraction and, for that very reason, is some sort of negation of real life... Science comprehends the thought of the reality not the reality itself; the thought of life, not life. That is its limit, its only really insuperable limit because it is founded on the very nature of thought, which is the only organ of science.

-Bakunin, God and the State

This thesis is based on the following papers which will be referred to in the text by their respective Roman numerals

- I A. Björklund and G. Skagerberg (1979) Simultaneous use of retrograde fluorescent tracers and fluorescence histochemistry for convenient and precise mapping of monoaminergic projections and collateral arrangements in the CNS. *Journal of Neuroscience Methods* 1: 261-277.
- II G. Skagerberg, A. Björklund and O. Lindvall (1984). Further studies on the use of True Blue as a retrograde fluorescent tracer in combination with monoamine histochemistry. Submitted to *Journal of Neuroscience Methods*.
- III A. Björklund and G. Skagerberg (1979) Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. *Brain Research* 177: 170-175.
- IV G. Skagerberg, A. Björklund, O. Lindvall and R.H. Schmidt (1982) Origin and termination of the diencephalo-spinal dopamine system in the rat. *Brain Research Bulletin* 9: 237-244.
- V G. Skagerberg and O. Lindvall (1984) Organization of diencephalic dopamine neurons projecting to the spinal cord in the rat. Submitted to *Brain Research*.
- VI O. Lindvall, A. Björklund and G. Skagerberg (1984) Selective histochemical demonstration of dopamine terminal systems in rat di- and telencephalon: new evidence for a dopaminergic innervation of hypothalamic neurosecretory nuclei. *Brain Research* 306: 19-30.
- VII G. Skagerberg, O. Lindvall and A. Björklund (1984) Origin, course and termination of the mesohabenular dopamine pathway in the rat. *Brain Research* 307: 99-108.
- VIII G. Skagerberg and A. Björklund (1984) Topographic principles in the spinal projections of serotonergic and non-serotonergic brainstem neurons in the rat. Submitted to *Neuroscience*.

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Abbreviations

Bb	Bisbenzimidazole
CA	Catecholamine
DA	Dopamine
DAPI	Diamidinophenylindol
DBH	Dopamine- β -hydroxylase
5,7-DHT	5,7-dihydroxytryptamine
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
DY	Diamidino Yellow
EB	Evans Blue
FB	Fast Blue
FITC	Fluorescein isothiocyanate
GB	Granular Blue
HRP	Horseradish Peroxidase
5-HT	5-Hydroxytryptamine
MA	Monoamine
NA	Noradrenaline
NY	Nuclear Yellow
6-OHDA	6-Hydroxydopamine
PI	Propidium Iodide
Pr	Primuline
TB	True Blue
TH	Tyrosine Hydroxylase
TRITC	Tetramethyl-rhodamine-isothiocyanate

Introduction

The present investigation is based on two important methodological breakthroughs in neurobiology, both of which were pioneered by Swedish investigators. The method for aldehyde-induced monoamine (MA) fluorescence was developed by Falck and Hillarp in the early sixties (Falck 1962, Falck et al. 1962) and permitted for the first time the direct visualization of neuronally localized monoamines. About ten years later Kristensson and Olsson were able to demonstrate that the fluorescent dye Evans Blue (EB) and the enzyme horseradish peroxidase (HRP) could, after having been injected in the region of nerve terminals, be retrogradely transported to the parent cell bodies where it could be detected by its fluorescence or enzymatic activity, respectively (Kristensson 1970, Kristensson et al. 1971).

Studies with the Falck-Hillarp method permitted the first visualization and mapping of the catecholamine-(CA) and indolamine-containing neuron systems in the mammalian CNS (Carlsson et al. 1962, Dahlström & Fuxe 1964, 1965, Fuxe 1965), and by using physical and chemical lesioning techniques the main features of the organization of central catecholamine and indolamine systems could be deduced (see Andén et al. 1966, Ungerstedt 1971). Later, the introduction of the glyoxylic acid method made possible a more complete and detailed mapping of the catecholamine systems, in particular (Lindvall and Björklund, 1974, 1978).

In 1978 when this thesis work was initiated, there were two major methodological shortcomings which prevented further progress in detailed anatomical studies on the MA systems. First, the new retrograde tracing techniques would not be really valuable for MA tracing unless they could be combined with fluorescence histochemistry on the same section. Thus, there was a need for a method which would allow simultaneous visualization of a retrograde tracer and visualization of the transmitter in the same cell bodies. In 1975 Ljungdahl et al. (1975) were the first to develop such a method, which allowed for detection of both HRP and tyrosine-hydroxylase (TH, the rate-limiting enzyme in the catecholamine biosynthetic pathway). Through this approach it became possible for the first time, to identify both the transmitter content and area of projection of individual CA neurons. Although this methodological development was of great importance, method was somewhat cumbersome to use since HRP and TH could not be visualized simultaneously without intervening processing. The second methodological shortcoming was the difficulty to distinguish dopamine (DA) from noradrenaline (NA) in the formaldehyde and glyoxylic acid methods. Although several fluorescence histochemical and immunohistochemical methods had been introduced for differentiation between dopaminergic and noradrenergic systems (Fuxe 1965, Andén et al. 1966, Butcher et al. 1972, Carlsson et al. 1965, Björklund et al. 1968, Hökfelt et al. 1973, 1975, Swanson and Hartman 1975) a differential distribution of the two amines in a given catecholaminergic terminal field could often only be hinted upon. Furthermore, the low levels of DA present in many NA-rich regions was often taken to indicate that the DA found was serving solely as a precursor for NA.

With the introduction of the fluorescent retrograde tracers True Blue (TB), Propidium Iodide (PI) and EB (Bentivoglio et al. 1979, Kuypers et al. 1977), new possibilities were opened to use retrograde tracers in direct combination with aldehyde-induced MA-fluorescence. The aims of the present investigation were, first, to develop such a method and apply it to the study of the organization of central



monoaminergic systems, and, secondly, to try to selectively visualize DA terminal systems in some regions within the mammalian CNS. For the latter purpose we chose to use selective monoamine neurotoxins which would remove virtually all NA innervation to particular regions, without affecting the DA innervation. For the previously undescribed systems the general strategy has been first to selectively visualize the DA fibres and map the distribution of the transmitter in the terminal area and thereafter to inject a fluorescent tracer in this region in order to establish the cell bodies of origin.

Material and methods

Animals

Throughout this study albino rats of the Sprague-Dawley strain were used (ALAB, Sollentuna).

Neurotoxin treatments

The following neurotoxic drugs were used: 6-hydroxydopamine (6-OHDA, Hässle AB), 5,7-dihydroxytryptamine (5,7-DHT, Regis) and N-(2-chloro-ethyl)N-ethyl-2-bromobenzylamine (DSP4, Astra AB). The substances were always administered freshly dissolved in saline. For removal of the spinal NA-innervation 6-OHDA was given neonatally on day 1 and 2 100 g/g, s.c. followed by intraventricular injections of 5,7-DHT (150 µg) at 2-3 months of age. In order to deplete forebrain NA four intracerebral injections of 6-OHDA (4 x 8 µg) were placed in the ascending NA bundles of adult animals who 5 and 10 days later received DSP 4 (50 mg/kg) subcutaneously. Intraventricular and intracerebral injections were performed under barbiturate anaesthesia (Brietal, Lilly, 40 mg/kg). (For more information about these neurotoxic drugs, see Johnsson 1983).

Tracer injections

All fluorescent substances were administered as water solutions or suspensions, and delivered through a Hamilton microsyringe either with a fitted coarse glass capillary (outer diameter 100 µm) or directly through the original Hamilton cannula. When a glass capillary was used the whole system was filled with paraffin-oil to avoid problems with compressible air. The large diameter of the capillary was necessitated by the high viscosity of, in particular, the TB suspension. A mechanical microdrive was attached to the Hamilton syringe to allow for slow and even delivery of tracer, usually at a rate of 0.01-0.02 µl/min. Injections into the brain or upper cervical cord were made stereotactically with the animal fixed in a Kopf stereotaxic frame. Injections at lower spinal levels were made under visual guidance with the microsyringe attached to a manipulator (Prior Instruments).

True Blue was purchased from Dr. Illing GmbH & Co KG and was used as a 2-3% suspension and sonicated for about one minute before use.

Histochemical procedure

Animals in which the serotonin (5HT) system was of main interest were pretreated with the monoaminooxidase-inhibitor Pargyline 200 mg/kg 2-5 hours before sacrifice. For perfusion the animals were deeply anaesthetized with chloral hydrate 50 mg/kg or barbiturate (Brietal) (40 mg/kg), i.p., and subsequently perfused through the ascending aorta, first with 150-200 ml ice-cold Tyrode's buffer followed by 300-350 ml of ALFA solution (Lorén et al. 1980) or, more often, a modified mixture containing 2 g paraformaldehyde + 5 g Al(SO₄) x 18 H₂O/100 ml of Tyrode's buffer with sodium acetate added to reach pH 4.7. The brain and spinal cord were rapidly removed, the right side sometimes marked with a drop of rhodamine solution, frozen in a liquid propane-propylene mixture, cooled to the temperature of liquid nitrogen and freeze-dried for five days at -35°C. The tissue pieces were then exposed to formaldehyde vapour at +80°C for 1 h (using formaldehyde which had been equilibrated with 50% relative humidity). The material was finally embedded in paraffin (melting point: +51-53°C) in vacuo and sectioned at 15 µm. In most cases every third section was mounted on glass-slides in an Entellan(Merck)-xylol mixture and coverslipped.

Sometimes adjacent sections were Nissl stained either with cresyl-violet or toluidine blue (for details of the fluorescence histochemical procedure see Björklund 1983).

Fluorescence microscopy.

Microscopical analysis was performed using a Zeiss Junior fluorescence microscope for transmitted light, using a HBO 200 mercury lamp (Osram) as light source. Three different excitation filters were used. Schott BG 12 (which has a high transmittance at the 405 and 435 nm peaks of the mercury lamp) was used for optimal visualization of catecholamine and serotonin fluorophores, Schott UG 1 (which transmits the 365 nm peak) for optimal visualization of TB and several of the other fluorescent tracers. A Schott AL 435 (which transmits the 435 nm peak) was used for selective visualization of monoamines with the exclusion of TB from the picture (see Hökfelt et al. 1983 for further details).

Catecholamine assay

DA and NA levels were determined in fresh tissue obtained from animals decapitated under ether anaesthesia. The amounts of the catecholamines were assayed radioenzymatically using a modification of the procedures described by DaPrada & Zürcher (1976) and Saller & Zigmond (1978). The method used in our laboratory has been described in detail by Schmidt et al. (1982).

Microspectrofluorometry

Spectral characteristics of the fluorescent tracers and MA fluorophores were recorded using a modified Leitz microspectrofluorometer as described by Björklund et al. (1972). All spectra were corrected for instrument factors according to the procedure described in that paper. Spectral recordings were obtained both from models (monoamines or tracer compounds dissolved in 2% bovine albumin solutions to a concentration of 0.1 mg/ml) and from tissue processed as described above. In the latter case the recordings were made using ordinary glass microscope slides and a glass condenser, as used for routine microscopy. Recording from models was carried out using an all quartz illumination system and quartz microscope slides.

Results

A. Combination of retrograde fluorescent tracers with aldehyde-induced monoamine fluorescence in freeze-dried material (papers I and II)

With the introduction of several substances that are fluorescent in their native condition and can be retrogradely transported in neurons (Kristensson 1970, Kuypers et al. 1977, 1979, Bentivoglio et al. 1979, 1980) it became possible to combine a retrograde tracer with the freeze-drying method for monoamine fluorescence. For such a combination to be useful it is required that the amine fluorophores and the fluorescent tracers can be selectively discerned in order to determine if individual cell bodies contain one or both types of compounds. Systematic investigations revealed that only three tracers had characteristics that could allow for them to be reliably differentiated against the monoamine fluorophores. This is most easily explained by referring to Fig. 1 which illustrates the differences in excitation characteristics for the different tracers and monoamine fluorophores. Differences in excitation characteristics have to us proved to be the most useful criterion for identifying monoamine fluorophores and tracers separately. Since, as shown in Fig. 1, the excitation light emitted from the mercury lamp is discontinuous narrow wavelength bands can easily be objectively chosen by varying the primary lamp filters. For practical purposes the 365, 405, 435, 546 and 577 nm peaks are the most useful since ordinary glass does not transmit wavelengths below 350 nm. As shown in Fig. 1, EB and PI are efficiently excited at 546 and 577 nm wavelengths which do not excite the monoamine fluorophores. TB, on the other hand, is not visualized at 435 nm excitation where the monoamines are easily seen. Thus, by changing excitation filters EB, PI and TB and the monoamine fluorophores can readily be differentiated from each other. An additional advantage of these tracers is that they have fluorescence colours which are markedly different from those of the CA or indolamine fluorophores. TB is thus more suitable than the closely related compounds granular blue, fast blue and DAPI both with respect to fluorescence colours and excitability at 435 nm. While differences in the wavelength of the fluorescence emission of the various substances (i.e. the fluorescence colour) also can serve for identification (Fig. 2) and are most valuable for microspectrofluorometric analysis we have, in case of TB, found this to be a less useful characteristic in routine microscopy, partly because the impression of colour is quite a subjective process and critically dependent on the optical characteristics of surrounding structures and partly because the colour sensitivity of the eye to longer wavelengths is increased as the fluorescence intensity increases (the so-called Bezold-Brücke effect).

The differential cellular distribution of different tracers has been profited from in studies concerned with collateralization (Van der Kooy et al. 1978, Kuypers et al. 1980) but are generally not very useful when using freeze-drying technique. In practice, however, both differences in fluorescence morphology, emission colour and excitation characteristics are taken advantage of though with emphasis on the last criterion.

Though both EB and PI may be used in conjunction with our standard method for aldehyde-induced monoamine fluorescence (paper I) they have some drawbacks which have limited their use in our laboratory. Thus, PI seems quite toxic, often inducing epileptiform discharges after intracerebral injections, and it is often so heavily deposited in cells that it is clearly seen also at 435 nm excitation at which

wavelength it may interfere with the identification of monoamines. While EB seems, at least acutely, non-toxic and does not interfere with the selective visualization of monoamines, both EB and PI fluorescent tracers diffuse rapidly from the injection site which becomes hard to demarcate. TB (see Fig. 3 for structural formula) does not have these disadvantages and we have therefore almost solely relied on this compound in our anatomical studies.

Our systematic investigations on the properties of TB (papers I and II) has shown this tracer to be ideally suited for tracing studies in combination with CA fluorescence. Thus, TB appears to be a very effective retrograde tracer, possibly with a sensitivity superior to HRP (Sawchenko & Swanson 1981), and TB can without interference be combined with monoamine fluorescence. The deposition of tracer both in the labelled cell bodies and at the injection site is very stable and the latter can be quite accurately separated into at least two different zones, the uptake from which has to some degree been determined. The stability of the injection site is an important prerequisite since optimal cell body labelling at sites distant from the injection site may require several weeks survival for optimal labelling. This is in sharp contrast to HRP. The injection site of this tracer changes very markedly during the first days after injection (Vanegas et al. 1978), and it disappears within a few days (Warr et al. 1981). This makes the optimum survival time for HRP shorter - about 1-2 days - but since HRP is transported more rapidly than TB, this time is sufficient for useful retrograde labelling. The differences in survival time required for optimal labelling with HRP and TB could reflect quite different mechanisms for uptake, transport and storage of the two compounds. We would, however, favor a unitary view in that both compounds use

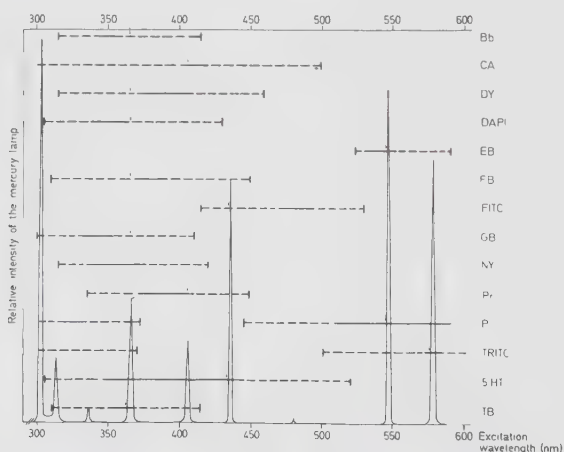


Fig. 1. In this figure the intensity of the different emission peaks of the mercury lamp, in the range between 300 and 600 nm, is indicated. The horizontal bars indicate the wavelengths at which the tracer compounds (indicated to the right) are efficiently excited. The dashed part of the bar indicates the range of 5-90% of maximum fluorescence intensity. The solid part of the bar indicates the wavelengths range at which the compound emits 90-100% of its maximum fluorescence. The arrows mark for each compound the mercury lamp peak which gives optimum excitation in the fluorescence microscope.

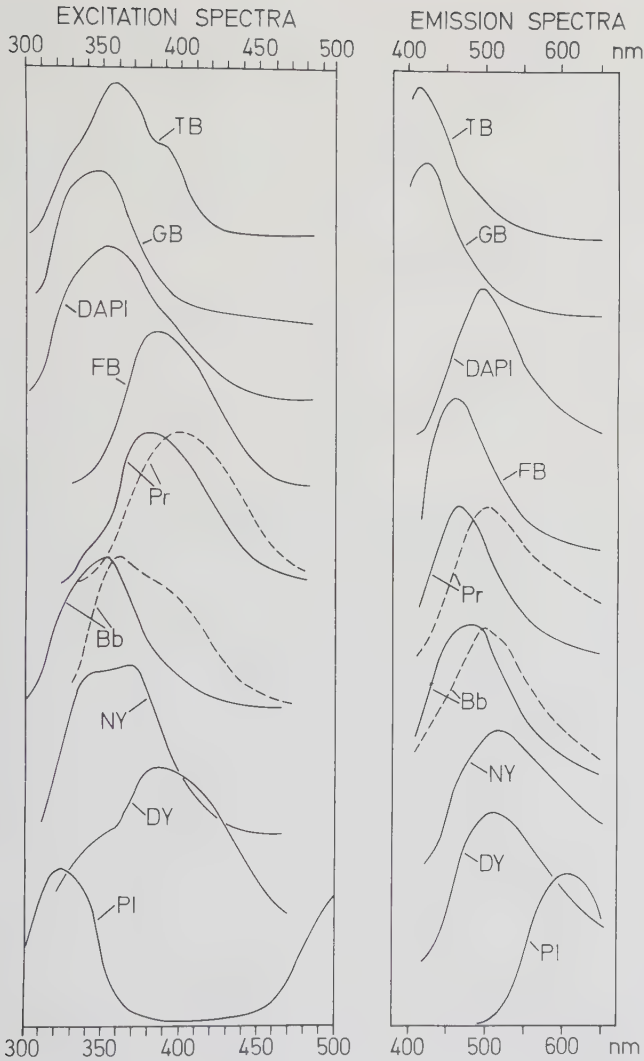


Fig. 2. Excitation and emission spectra for some fluorescent tracers in models. In the excitation spectra to the left the height of the curve indicates the efficiency of fluorescence excitation at the corresponding wavelength. The emission spectra indicate the intensity of the emitted light when the tracer is excited at its maximal excitation wavelength. Measurements were performed on the compounds enclosed in a dried protein matrix as described in paper I. For primuline and bisbenzimidazole the spectra recorded in tissue processed according to the procedure described in the material and methods section.

True Blue

trans-1,2-bis (5 amidino-2-benzofuranyl)etylenedihydrochloride

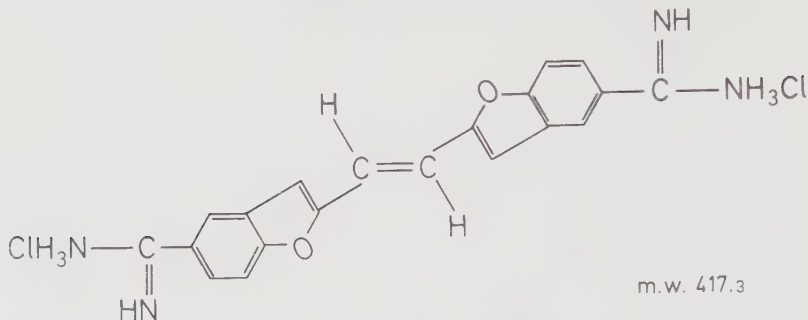


Fig. 3 Structural formula and molecular weight for the fluorescent retrograde tracer True Blue.

similar or identical mechanisms for uptake by unspecific fluid phase endocytosis (Zacks & Saito 1968, Edelson & Cohn 1978), that they both use retrograde transport by several different systems (Grafstein & Forman 1980) and storage in the lysosomal compartment of the cell body (Chu-Wang & Oppenheim 1980). The differences encountered between the various tracers may then rather reflect differences in the efficiency of uptake, transport and storage and variations in levels required for detection or in degree of intracellular breakdown.

Though we have found the combined TB-monoamine fluorescence method described both sensitive and specific some caution should be exercised when evaluating both positive and negative results obtained by this technique. While the method rests on the dogma that the transmitter present in the cell body is also functioning in the terminal areas of the cell it is conceivable that (perhaps especially in the cases of catecholaminergic systems), the particular transmitter synthesized in the cell body could be modified by local influences in the terminal area. Thus, for example, retrograde labelling in a dopaminergic cell body from a tracer injection in a terminal area, though being very suggestive does not prove dopaminergic transmission in the area of tracer injection. Indeed it has been shown that individual collaterals of the same neuron may exhibit synaptic vesicles of different morphology possibly indicating different synaptic mechanisms at the different terminals (Triller & Korn 1981). Furthermore, there are several examples showing that cellular CA synthesis can be under regulation from the local environment. Thus, it has been shown that the extent to which adrenal medullary cells will synthesize and store adrenaline and NA is regulated both hormonally (by corticosteroid action) and neuronally (by the activity of the autonomic innervation of the cells) (see Wurtman et al. 1972, Thoenen 1975). Also, during development, the ability of sympathetic ganglionic neurons to synthesize NA is dependent on influences from the environment, including activity in the preganglionic nerves (see Patterson 1981).

Failure to retrogradely label a particular set of neurons, on the other hand, should not be equaled with absence of terminals of these neurons at the injection site. Thus, we have some evidence (paper V) that when the injection covers only a small fraction of the total

terminal area of a neuron there may be no detectable retrograde labelling in the cell body. In such cases the degree of neuronal activity may be the decisive factor as to whether retrograde labelling will be seen or not. In most cases, however, the degree of neuronal activity is probably of less importance (Enerbäck et al. 1980). It must also be realized that with appropriate selection of filter-thickness, TB labelling in MA-containing neurons will usually be readily shown by the increased fluorescence intensity as the excitation light is shifted from 435 to 365 nm. This is not necessarily an all or none phenomenon. One would guess that in most cases there are at least some neurons with so low degree of tracer labelling that the presence of TB would be suggested only by e.g. a less marked decrease in fluorescence at 365 nm as compared to that seen in unlabelled MA neurons.

B. The diencephalo-spinal DA system (papers III, IV, V)

The presence of DA in the spinal cord was acknowledged already in the earliest studies concerned with the distribution of catecholamines within the CNS. The amounts of DA were however very low compared to the NA-levels and therefore it was generally assumed that the spinal DA did only serve as a precursor to NA. A clear indication as to the possibility of a independent spinal DA innervation came from the work of Magnusson (1973) who could demonstrate that spinal DA and NA did not disappear concomitantly below a spinal transection. Despite this suggestive finding it was not until 1979 that we as well as two other groups could present morphological evidence for such a system (Blessing & Chalmers 1979, Hökfelt et al. 1979) originating from the A11 cell group.

In order to clarify the distribution of the spinal DA innervation rats were subjected to neurotoxin treatment both neonatally (6-OHDA systemically) and as adults (5,7-DHT intraventricularly). Chemical measurements of spinal NA and DA levels showed that this treatment caused a very marked reduction of NA while the DA levels were affected only to a very limited extent. This led to an increase in the ratios of DA to NA, in dorsal and intermediate regions of the cord, from less than 0.15 in normal animals to about 1.5 in intermediate regions and to about 5 in more dorsal areas. Since the fluorescence yield of DA is as good if not better as that of NA (Björklund et al. 1980) it can be safely assumed that most of the CA fluorescence seen in the spinal cord after this neurotoxin treatment represents DA. Fluorescence microscopical analysis of spinal cord tissue from neurotoxin treated rats revealed that the remaining CA fluorescence was almost exclusively found in the intermediate zone and in the dorsal horn and dorsal part of the lateral funiculus. Fluorescent fibres in the intermediate zone could be seen throughout the length of the cord but were most conspicuous at thoracolumbar levels where fibre strands could often be seen between the intermediolateral cell column and the area around the central canal. Fluorescent fibres could be seen throughout the dorsal horn but seemed concentrated to its dorsolateral part and in the reticular nucleus (i.e. lateral parts of lamina V).

After injections of fluorescent tracer into the spinal cord of neurotoxin-treated and normal rats the diencephalic A11 cell group (Björklund & Nobin 1973) was the only area known to contain DA cells that became retrogradely labelled (paper V). Since many cells in this area are TH positive (Hökfelt et al. 1976) while none appears to contain dopamine- β -hydroxylase (Swanson & Hartman 1975) it follows that the retrogradely labelled CA-fluorescent cells in this area seen in our studies should constitute the cell bodies of origin for the

spinal DA system. By placing the tracer injections at different levels and in different parts of the cord we tried to unravel the organization of the system. We were in particular curious as to obtain evidence for or against a topographical arrangement of the system and to see if this system showed signs of being collateralized. No evidence for a topographical organization of the system was found, however. Instead, our findings of a similar number and distribution of retrogradely labelled A11 cells after injections above, at and below the level of the IML, indicates a mode of organization of DA neurons where most, if not all axons traverses the whole terminal area (i.e. the spinal cord) while probably giving of collaterals at several levels. In this respect the A11 DA neurons seem to differ from what has been proposed to be the general projection principles of DA neurons elsewhere in the brain (see Björklund and Lindvall 1984).

Performing the same kind of numerical exercise as in paper VII we may assume that the whole spinal cord of the rat contains about 10 ng DA (20 ng/g according to Magnusson 1973 and calculating with a cord weight of 0.5 g). Since the average total DA content of a single DA neuron in the rat brain has been estimated to be approximately 30 pg (Björklund & Lindvall 1984) about 300 DA neurons would be required to supply the cord with its DA innervation. Our finding of about 40 labelled DA-containing A11 cells after hemichord injections are thus less than a third of the expected number. There are at least three possible explanations for this apparent misfit. (i) First it must be emphasized that the estimated DA content per cell is really very approximate and also that the spinal projecting DA neurons could certainly contain more DA than the ones having more localized projections. (ii) It is possible that some DA neurons could be so weakly labelled that the DA fluorescence could effectively conceal the presence of tracer (c.f. the last paragraph in A). (iii) Some of the spinal projecting cells (e.g. in the A11 area) that we have classified as non-DA may in fact synthesize DA but at too low levels (at least in the cell body) for allowing its detection with the aldehyde-induced fluorescence method. Such a proposition is to some extent validated by the immunocytochemical finding of fairly large numbers of TH-containing neurons in the hypothalamus that are not visualized by the aldehyde-induced fluorescence technique (Hököfelt et al. 1976, 1984). Through the courtesy of Professor Tomas Hököfelt, Stockholm, we have had the opportunity to examine TH-stained sections through some hypothalamic levels which showed that the numbers, location and morphology of large TH-positive neurons in this region are such that this population could possibly encompass most of the spinal projecting cells in the A11 area.

The functional role of spinal DA is still obscure though its restricted distribution to dorsal and intermediate areas would implicate an involvement in sensory and autonomic mechanisms. There is some experimental evidence to support this notion, for example it has been shown that DA can, via a spinal mechanism, increase renal sympathetic nerve activity (Simon & Schramm 1983) and also that stimulation of spinal DA receptors may diminish the response to painful stimuli (Jensen & Yaksh 1984). While the physiological significance of these findings is still unclear it seems possible that some of the symptoms seen in Parkinsons disease as well as some of the effects of the commonly used DA-receptor blockers could be attributed to an effect on the spinal DA-system.

C. Selective histochemical demonstration of dopamine terminal systems in rat di- and telencephalon (paper VI)

As mentioned in the introduction, a major obstacle to the study of central catecholaminergic systems has been the inability of the methods based on aldehyde-induced fluorescence to selectively visualize DA or NA. The two catecholamines have an overlapping distribution in several CNS areas with a high NA to DA ratio and this has sometimes erroneously led to assigning to DA only a precursor role in these regions. By using antibodies against the enzyme dopamine- β -hydroxylase (DBH, which converts DA to NA) it became possible to selectively visualize NA synthesizing fibres. By correlating the distributional pattern thus obtained with that reported through the use of aldehyde-induced fluorescence (see e.g. Lindvall & Björklund 1974), Swanson and Hartman (1975) could suggest that some areas which contain CA terminals (e.g. the lateral habenula) in fact receives DA fibres only. The problem remained, however, unsolved for areas containing both DA and NA terminals, awaiting a method for the positive selective identification of DA systems. We accomplished to develop such a method by taking advantage of an additive effect of two neurochemical lesioning techniques. In adult animals 6-OHDA was injected bilaterally into the main ascending NA-fibre bundles, followed by systemic injections of the NA-selective neurotoxin DSP4 (Jonsson et al. 1981). Chemical measurements revealed that this treatment, while leaving DA levels virtually unaffected, produced a very marked depletion of NA in di- and telencephalic areas. In the diencephalon NA was reduced by 86-96%, and in telencephalic regions the reduction NA was even more pronounced. In such animals the ratio of DA to NA in the areas examined was increased to such a degree (to 1.6-9.1) that most of the fibres seen with the aldehyde-induced fluorescence should be DA-containing. In addition to confirming the presence of DA in regions which previously had been suggested to receive DA fibres (e.g. the lateral habenula) previously undemonstrated DA innervations could be demonstrated in the supra-optic, paraventricular and dorsomedial nuclei of the hypothalamus and in the thalamic paraventricular nucleus. It should be noted though that Fuxe already in 1965 (Fuxe 1965) suggested that some of these areas could contain DA innervations. The functional significance of these "new" areas of DA innervation is presently unknown but the presence of DA terminals in magnocellular hypothalamic nuclei certainly points to a more widespread role of DA in neuroendocrine regulation than has previously been suspected. In particular; Moos and Richard (1982) have suggested that hypothalamic DA may regulate the release of vasopressin in response to osmotic variations and also to facilitate the reflex release of oxytocin in response to suckling. With the exception of the DA innervation of the lateral habenula (see below) there is no information available concerning the origin of the diencephalic DA-terminal fields. Although it seems reasonable to expect them to be supplied by intradiencephalic DA cell bodies our results from preliminary tracing experiments does not support this notion.

D. The mesohabenular DA system (paper VII)

In a first attempt to arrive at a more detailed knowledge of the anatomy of diencephalic DA systems we studied the origin, course and termination of the DA system innervating the habenula. With the modified ALFA technique the habenula two distinctly separate types of catecholaminergic fibres were seen in this nucleus. In the medial habenula thick, very intensely fluorescent fibres are common, these

have been shown to contain NA and to be of sympathetic origin, (Björklund et al. 1972). The second type of fibre is much more delicate and distributed exclusively in the lateral subnucleus where these fibres form a dense aggregation in the medial part of the caudal two thirds of the nucleus. The dopaminergic nature of this fibre cluster in the lateral habenula is substantiated by several independent lines of evidence. (i) Immunohistochemical observations has shown that while this nucleus contains many TH-positive fibres (Hökfelt et al. 1976) there are only very few DBH positive ones (Swanson & Hartman); (ii) chemical measurements after microdissection demonstrated higher levels of DA than NA in the lateral habenula (Phillipson & Pycock 1982); (iii) removal of all major NA inputs left this innervation intact (Lindvall et al. 1974); (i.v.) uptake experiments (in paper VII) showed that these fibres possess a desipramine resistant capacity for DA uptake.

There was an almost complete disappearance of fluorescent fibres in the lateral habenula after knife-cuts through the fasciculus retroflexus, whereas a similar lesion of the stria medullaris did not seem to remove any of these fibres. Our lesion data thus demonstrated that the axons giving rise to the DA innervation of the lateral habenula reach this nucleus by passing within or along the fasciculus retroflexus. Since this tract passes through the ventral mesencephalon it seemed highly probable that the cell bodies of origin of the habenular DA system should be located in the ventral tegmental area which is known to contain large numbers of DA cell bodies. Moreover, previous HRP studies had demonstrated a limited projection from this area the lateral habenula (Herkenham & Nauta 1977, Phillipson & Griffith 1980). To test this hypothesis TB was injected into the region of DA innervation in the habenula and the distribution of retrogradely cell bodies were mapped. The only labeled DA neurons found were indeed situated in the ventral tegmental area, most of them being located in or close to the ipsilateral so-called interfascicular nucleus. An unexpected finding was that the majority of the retrogradely labeled cells in this area were, however, apparently non-dopaminergic.

Though the functional role of the habenula is known only to a very limited extent (see Sutherland 1982) its strategic location along the output pathway from limbic, hypothalamic and striatal regions taken together with its known projections to e.g. the dorsal raphe and substantia nigra (Herkenham & Nauta 1979) in e.g. ascending 5HT systems and via a feed-back loop could also regulate mesencephalic DA-cell activity.

E. Spinal projections from brain stem regions containing serotonergic neurons (paper VII)

Spinal projections from the medullary raphe nuclei were established by Brodal et al. already in 1960 (Brodal et al. 1960). With the findings of Dahlström and Fuxe (1964, 1965) that many cells in this region contain 5HT, and that 5HT-containing axons and terminals are present in the spinal cord, it was logically assumed that the medullary raphe was the origin of the spinal 5HT projections. Much interest was focused on this system when it became clear that the spinal serotonin system was important for descending inhibition mediating analgesia (see, e.g., Yaksh and Wilson 1979). It was gradually realized, however, that the raphe spinal system also contained non-serotonergic components. Thus, Wiklund (1981) could document the presence of large numbers of non-5HT-containing neurons among the serotonergic

ones and West and Wolstencroft (1977) demonstrated some raphe-spinal neurons with conduction velocities not compatible with them not being serotonergic. On the other hand, already Dahlström and Fuxe (1964) had shown that 5HT cells also were distributed outside the raphe nuclei. We decided to apply the combined retrograde tracer + aldehyde-induced fluorescence technique to study in more detail the location of the cells within or close to the raphe nuclei that projects to the spinal cord and to determine which of these were 5HT-containing. For this purpose TB was injected at different levels and in different regions in the spinal cord and the distribution of TB-labelled serotonergic and non-serotonergic neurons was mapped in areas containing 5HT cells in the brain stem. Through this approach it was possible to establish the main features of the organisation of these descending systems and also to quantify the serotonergic and non-serotonergic components.

Our results were generally in agreement with other studies which had indicated that the rostral portion of the medullary raphe region projects to dorsal regions throughout the length of the cord while the more caudally situated cell bodies projected mostly to the ventral horn (Basbaum et al. 1978, Martin et al. 1978), and also demonstrated that such an orderly arrangement was most pronounced for the 5HT neurons.

A striking finding was the observation of large numbers of spinal-projecting non-5HT neurons intermingled with serotonergic cells in all areas studied. This was especially prominent at the level of the nucleus raphe magnus and surrounding area, corresponding to the nucleus reticularis gigantocellularis pars α in which the non-5HT cells were about 3-4 times more numerous but less pronounced in e.g. nc. raphe pallidus and obscurus. With respect to the number of spinal projecting 5HT and non-5HT neurons such estimates are of course critically dependent on how the areas examined are defined and therefore comparisons with other studies must be performed with caution.

When comparing the data obtained for 5HT and non-5HT neurons projecting to the spinal cord from the region studied it appears that the 5HT neurons exhibit a high degree of topographical arrangement while the non-5HT cell bodies projecting to a certain sub-region of the cord are generally more widely distributed. Nevertheless our data speaks in favour of classifying all the spinal projections from these areas as belonging to one of three systems that we have designated the dorsal, intermediate or ventral pathway, respectively. The dorsal pathway takes its origins mainly from cells in the caudal pons and rostral medulla, descends in the dorsal part of the lateral funiculus and terminates mainly in the dorsal horn. This system contains both serotonergic and non-serotonergic neurons, but the non-serotonergic ones predominate. This is the pathway involved in mediating descending inhibition upon spinothalamic tract neurons (see e.g. Watkins & Mayer 1982). Many of the serotonergic neurons in this system seems highly collateralized and the observation that a part of this projection terminates in the intermediate zone makes it tempting to speculate that neurons projecting through this pathway and terminating both within the intermediate zone and dorsal horn could serve as an anatomical substrate for the reported correlation of elevated blood pressure and analgesia (Zamir & Segal 1977). The intermediate pathway originates mainly from serotonergic neurons in the arcuate cell group. This cell group which is made up of a large number of small cells located very close to the ventral surface of the brainstem are peculiar not only with respect to their location and morphology but also in that their 5HT content appears quite variable. Thus, it is difficult to say whether there are indeed any non-5HT neurons in this cell

group. The descending axons in this pathway descends through the lateral funiculus and terminates largely in the region of preganglionic autonomic neurons. The ventral pathway with its 5HT and non-5HT components, descends through the ventral funiculus and gives off terminals to the ventral horn mediating brainstem influences upon somatic motoneurons. In comparison with the 5HT cells which projects through the dorsal pathway the 5HT neurons that course in the ventral and intermediate pathways shows signs of a lower degree of collateralization and are also generally somewhat smaller. Our study also demonstrates that there most likely are also ascending projections from the 5HT cells in the caudal brainstem, thus physiological effects elicited from stimulation in this area must not necessarily involve spinal mechanisms.

Concluding remarks

The neurotransmitter-specific retrograde tracing technique which has been developed in this study has, together with the described method for selective NA-depletion, revealed several new features of central MA systems. In several areas previously known to receive only one type of CA innervation, namely NA, firm evidence have been presented also for separate DA innervations which, though quantitatively small have interesting functional implications.

In comparison with other central DA systems the diencephalo-spinal DA system exhibits a new mode of organization in which a high specificity in terms of origin and region of termination is combined with a high degree of collateralization along a zone of functionally similar areas. Possibly such a design could implicate a great need for synchronous activation. In contrast, the complex organization of the descending serotonergic system points to the many possible variations within a monoamine projection system that otherwise may be looked upon as a functional unit.

The finding of non-MA neurons in classical MA cell areas is not new but the high degree to which it has been shown to occur underlines the importance of neurotransmitter characterization in conjunction with retrograde tracing.

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SIMULTANEOUS USE OF RETROGRADE FLUORESCENT TRACERS
AND FLUORESCENCE HISTOCHEMISTRY FOR CONVENIENT
AND PRECISE MAPPING OF MONOAMINERGIC PROJECTIONS
AND COLLATERAL ARRANGEMENTS IN THE CNS

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SIMULTANEOUS USE OF RETROGRADE FLUORESCENT TRACERS AND FLUORESCENCE HISTOCHEMISTRY FOR CONVENIENT AND PRECISE MAPPING OF MONOAMINERGIC PROJECTIONS AND COLLATERAL ARRANGEMENTS IN THE CNS

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A procedure is described for the use of fluorescent retrograde tracers in conjunction with monoamine fluorescence histochemistry for single or double labeling studies of the projections of identified catecholamine or indoleamine neurons in the CNS. A number of fluorescent tracers, including Evans Blue, bisbenzimidazole, DAPI, 'True Blue', 'Granular Blue' and propidium iodide, have been tested and characterized microspectrofluorometrically. Of these, 'True Blue', propidium iodide and Evans Blue were found to have the most suitable properties to be studied concomitant with the monoamine fluorophores. A combination of two of these tracers — 'True Blue' fluorescing blue and propidium iodide or Evans Blue fluorescing orange to red — and the catecholamines and indoleamines fluorescing yellow-green and yellow to brownish yellow, respectively, makes possible simultaneous use of four different fluorescent markers in one and the same section. The present procedure has great practical advantages over available HRP techniques and should in modified form also be applicable to other types of transmitter-specific neuronal tracing based on immunocytochemistry or enzyme histochemistry.

INTRODUCTION

Detailed mapping of projections of monoamine-containing neurons in the CNS would be greatly facilitated by microscopic methods allowing the use of retrograde axonal tracer substances concomitant with monoamine fluorescence histochemistry. Procedures using horseradish peroxidase (HRP) for this purpose have recently been published (Berger et al., 1978; Blessing et al., 1978). Since they involve restaining of the fluorescence histochemical sections with re-photography of each section area for cell identification, the combined HRP procedures suffer, however, from being cumbersome and time-consuming.

An entirely new possibility was recently opened up by Kuypers and his collaborators (Kuypers et al., 1977, 1979; Van der Kooy et al., 1978; Bentivoglio et al., 1979) through the introduction of fluorescent retrograde tracers. If properly selected, such tracers should be possible to visualize

concomitant with the catecholamine or indoleamine fluorophores in the fluorescence microscope. In the cited studies Kuypers and collaborators used frozen sections of formaline-fixed material, a mode of processing which is unsuitable for monoamine histochemistry. In the present study we have tested the possibility of using fluorescent retrograde tracers in freeze-dried, paraffin-embedded material processed for sensitive monoamine visualization according to the standard Falck-Hillarp method or the aluminum-formaldehyde (ALFA) method of Lorén et al. (1979), and we have selected the tracers which have the most suitable properties to be studied concomitant with the monoamine fluorophores. Of the fluorescent tracers tested — Evans Blue, bisbenzimidazole, DAPI, 'True Blue', 'Granular Blue' and propidium iodide — the results show that Evans Blue, 'True Blue' and propidium iodide are most ideally suited for this purpose, and that these compounds can be applied for convenient and detailed tracing of monoaminergic and non-monoaminergic projections in the CNS.

MATERIALS AND METHODS

The following compounds were tested: Evans Blue (Sigma; 10% w/v solution); 4,6-diamidino-2-phenylindol-2HCl (DAPI; Serva, Heidelberg; 2.5% w/v solution); bisbenzimidazole (33258 Hoechst¹; 10% w/v solution); trans-1,2-bis(5-amidino-2-benzofuranyl)ethylene-2HCl ('True Blue'²; 5% w/v solution); 2-[4-[2(4-amidinophenoxy)ethoxy]phenyl]indol-6-carboxamidin-2-HCl ('Granular Blue'²; 5% w/v solution); propidium iodide (Sigma; 0.75% suspension). The compounds were dissolved or suspended in distilled water and injected in volumes of 0.1–2.0 μ l. Some of the injection solutions were stored in a freezer (–20°C) for several months without any notable loss in fluorescence intensity or retrograde labeling efficiency. The injections were made into the head of the caudate-putamen and into the cervical spinal cord of young adult Sprague-Dawley rats (180–200 g body weight) under barbiturate (Brietal, Lilly, 40 mg/kg) or combined Ketalar (Parke-Davis, 10 mg i.m.) and Rompun (Bayer, 2 mg i.m.) anesthesia.

After 3–7 days survival the brains were either processed, without perfusion, according to the Falck-Hillarp method (see Björklund et al., 1972) or perfused and processed for monoamine fluorescence histochemistry according to the aluminum-formaldehyde (ALFA) method of Lorén et al. (1979). In short, the latter method involves the perfusion of the rat with first, 150 ml of ice-cold Tyrode's buffer, pH 7.0, followed by 300 ml of the same ice-cold buffer containing 10 g $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$ /100 ml and 2% paraformaldehyde (final pH about 3.8). The brains and spinal cords were then rapidly dissected, frozen in a liquid propane-propylene mixture cooled to the tem-

¹ 33258 Hoechst was kindly supplied by Dr. H. Loewe, Hoechst A.G., Frankfurt, G.F.R.

² 'True Blue' (compound 150/129) and 'Granular Blue' (compound 186/134) were kindly supplied by Dr. O. Dann, Institut für Pharmazie und Lebensmittelchemie der Friedrich-Alexander-Universität, D 8520 Erlangen, G.F.R.

perature of liquid nitrogen, and then freeze-dried at -35°C . The freeze-dried tissue pieces were reacted with gaseous formaldehyde at $+80^{\circ}\text{C}$ for 1 h (using paraformaldehyde equilibrated in 50% relative humidity) and finally embedded in paraffin in vacuo (for details on the preparative procedure, see Björklund et al., 1972).

The injection sites and the brain stem regions were serially sectioned at about $20\text{ }\mu\text{m}$ and every 2nd to every 4th section was mounted in Entellan (Merck, Darmstadt, G.F.R.) containing about 20% xylene to dissolve the paraffin (cf. Björklund et al., 1972). The section were studied under a Zeiss Junior fluorescence microscope, equipped with a HBO 200 mercury lamp, or, in case of Evans Blue and propidium iodide injected specimens, under a Leitz Orthoplan microscope equipped with the Ploem-Opak illuminator (filter-mirror system N_2). In the Zeiss microscope the following filter combinations were used: for excitation with the 365 nm line of the mercury lamp, Schott UG 1 as lamp filter plus Zeiss 41 and 65 as secondary barrier filters; for excitation with 405 nm line, Schott BG 12 as lamp filter plus Zeiss 44 and 47 as secondary barrier filters; for excitation with the 435 nm line, Leitz AL 435 band interference filter as lamp filter and Zeiss 44 and 47 as secondary barrier filters.

Spectral recordings and fluorescence intensity measurements were made in a Leitz microspectrofluorometer as described elsewhere (Björklund et al., 1972). All spectra were corrected for instrument factors according to the procedures described in that paper. In the model experiments the various compounds, as well as dopamine, noradrenaline and serotonin, were dissolved in aqueous 2% albumin solutions to a concentration of 0.1 mg/ml. Droplets of $0.1\text{ }\mu\text{l}$ were spotted onto glass coverslips, and after drying the droplets were exposed to formaldehyde vapour at $+80^{\circ}\text{C}$ for 1 h. In models, the fluorescence excitation and emission spectra were recorded using an all-quartz illumination system and quartz microscope slides. In tissue sections, the recordings were made with glass microscope slides and glass condensor, as used in routine microscopy.

RESULTS AND COMMENTS

A fluorescent tracer would be useful for study in combination with monoamine fluorophores if it were distinguishable from these fluorophores either (a) by having a *different fluorescence color* (i.e. *different emission spectrum*), or (b) by having a *different excitation (activation) maximum*, or (c) by having a *different subcellular distribution within the labeled perikarya*. Below, each of these criteria are evaluated for the tracers tested.

(A) Appearance of retrogradely labeled neuronal perikarya

All compounds tested, Evans Blue (EB), DAPI, 'True Blue' (TB), 'Granular Blue' (GB), bisbenzimidazole and propidium iodide (PI), were very well

demonstrable in neocortex, the centromedian-parafascicular nucleus, and substantia nigra after injection into the caudate-putamen, and in spinal cord-projecting neurons in the brain stem and the sensorimotor cortex after injection into the cervical spinal cord. Injections of EB into the cervical spinal cord resulted in clear-cut labeling only in cell bodies of the lower brain stem. The general distribution of labeled cells thus conforms to the previous descriptions of Kuypers et al. (Kuypers et al., 1977, 1979; Bentivoglio et al., 1979a, b). In agreement with their findings the most intense cell body label-

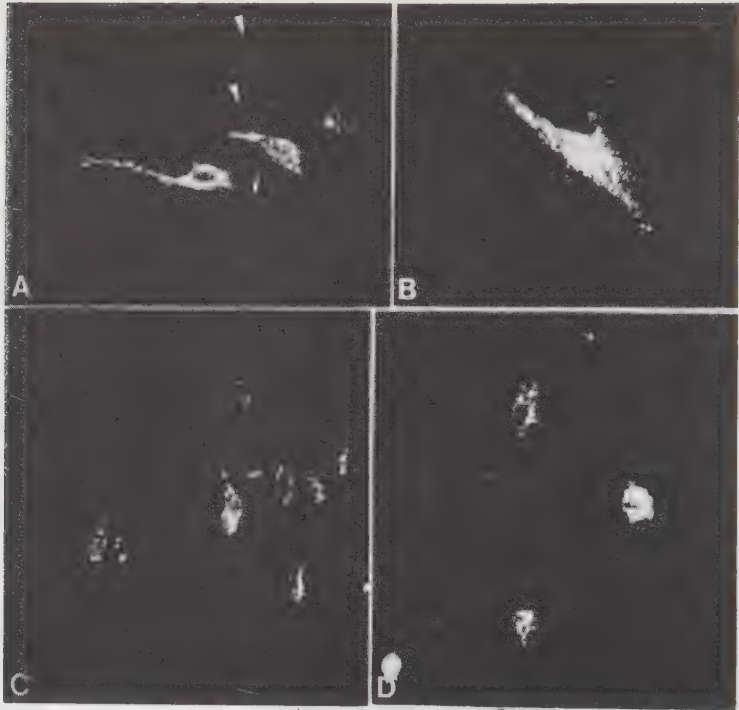


Fig. 1. Appearance of labeled cell bodies. **A:** in the pontine reticular formation after injection of PI into the cervical spinal cord (3 days survival, illumination at 546/577 nm; $\times 350$). Note the presence of PI in the delicate axon (arrow) and in the dendrites. Arrow heads mark weakly labeled glial cell nuclei. **B:** in the centromedian-parafascicular nucleus after injection of DAPI into the nc. caudatus-putamen (3 days survival, illumination at 365 nm; $\times 500$). **C:** in the sensorimotor cortex after injection of GB into the nc. caudatus-putamen (3 days survival, illumination at 365 nm; $\times 290$). **D:** in the sensorimotor cortex after injection of bisbenzimidazole into the cervical spinal cord (7 days survival, illumination at 365 nm; $\times 290$).

ing occurred with bisbenzimidazole, DAPI, TB and GB. Bisbenzimidazole, GB and PI also labeled axons of retrogradely labeled neurons, e.g. in the nigrostriatal pathway.

In the freeze-dried specimens all tracers except EB were confined to granules in the cytoplasm of the labelled neuronal cell bodies (Figs. 1–4). In frozen sections of formaldehyde-fixed tissue both DAPI (Van der Kooy et al., 1978; Kuypers et al., 1977), TB (Bentivoglio et al., 1979b), bisbenzimidazole and PI (Kuypers et al., 1979) give mostly a diffuse cytoplasmic labeling, as well as nuclear or nucleolar staining. Clear-cut nucleolar staining was in the present freeze-dried material seen only in PI-labeled cell bodies (Fig. 3D). The most likely explanation of these differences is that freeze-drying at low temperature (-35°C) preserves the *in vivo* localization of the compounds better than the frozen sectioning does (cf. Stumpf and Roth, 1969). Furthermore, storage of the tissue in sucrose buffer before sectioning, as employed by Kuypers and collaborators (Van der Kooy et al., 1978; Kuypers et al., 1979) may allow for subcellular redistribution of the tracer substances. With bisbenzimidazole and PI, and to a lesser degree with TB and GB, nuclear staining was abundant in glial cells along the axon pathways and in the vicinity of

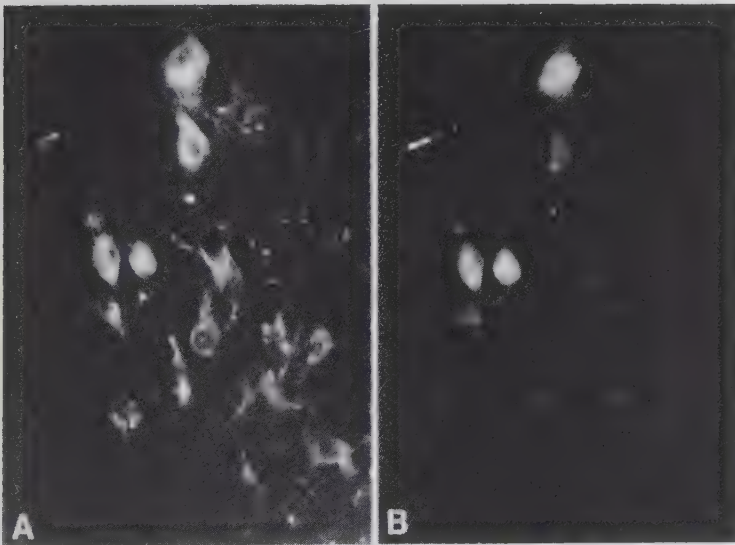


Fig. 2. Concomitant visualization of dopamine and EB in the pars compacta of the substantia nigra after EB injection into the nc. caudatus-putamen (3 days survival). A: activation at 405/435 nm showing selectively the cytoplasmic, yellow-green formaldehyde-induced dopamine fluorescence. B: activation at 546/577 nm showing selectively the diffuse, red EB fluorescence in some of the dopamine-containing cells ($\times 300$).

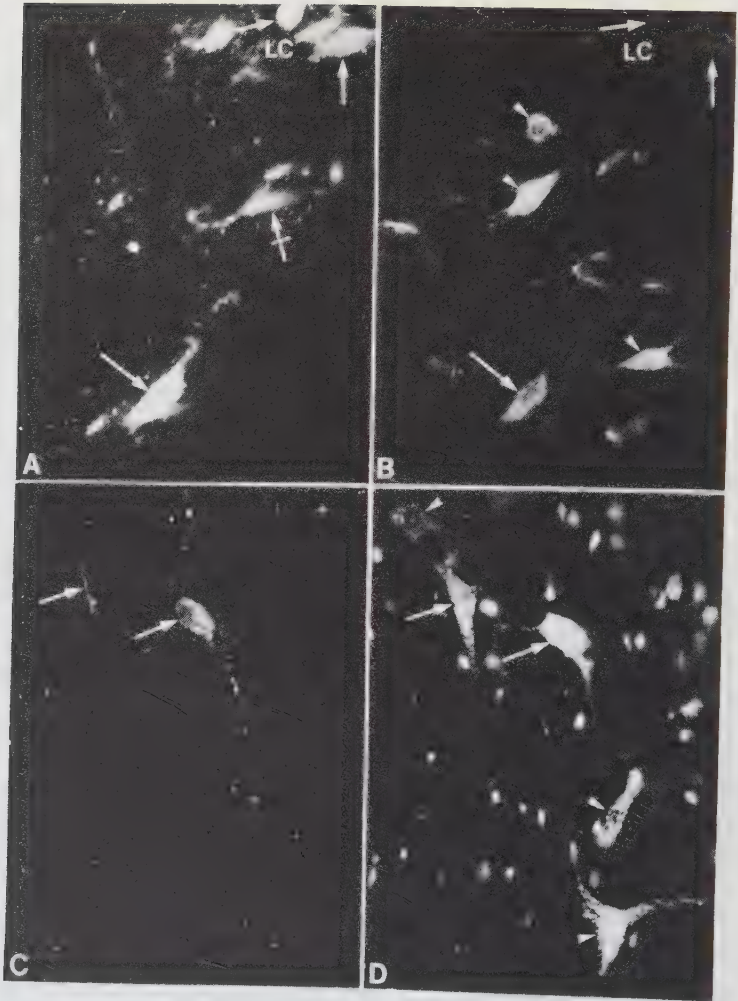


Fig. 3. Concomitant visualization of monoamines and PI in the brain stem after injection of PI into the cervical spinal cord, 3 days survival. A and B are from the locus coeruleus (LC), subcoeruleus region, and C and D from the lateral part of the nc. raphe magnus. A and C: the yellowish noradrenaline fluorescence (A) and the brownish-yellow serotonin fluorescence (C) is selectively visualized at 405 nm activation, B and D: the granular orange-red PI fluorescence is selectively visualized at 546/577 nm activation. Arrows denote noradrenaline- and serotonin-containing cell bodies which also contain the PI tracer. Crossed arrow in A indicates part of a non-labeled noradrenaline-containing cell body. Arrowheads denote non-monoaminergic PI labeled cell bodies. In D many PI-fluorescent glial cell nuclei are present ($\times 325$).

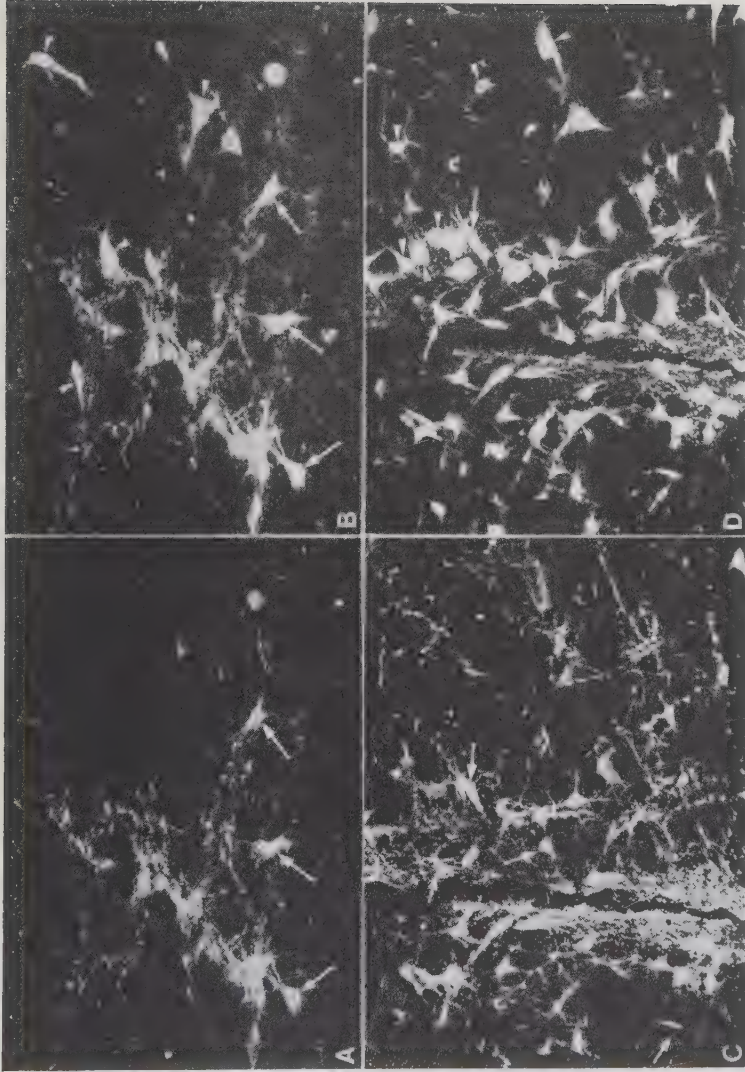


Fig. 4. Concomitant visualization of monoamines and TB in the brain stem after injection of TB into the cervical spinal cord, 7 days survival. A and B: from the area of the A1 catecholamine cell group. C and D: from the medial part of the nc. raphe magnus. In A the yellowish catecholamine (presumably noradrenaline) fluorescence, and in C the brownish-yellow serotonin fluorescence, is selectively visualized at 435 nm activation. In B and D the granular, ice-blue TB fluorescence is present together with the cytoplasmic monoamine fluorescence at 365 nm activation. Arrows denote catecholamine- and serotonin-containing cell bodies which contain the TB tracer, too. Arrowheads indicate TB-labeled cell bodies which are non-monoaminergic. It is evident that both the A1 cell group and the nc. raphe magnus contain both monoaminergic and non-monoaminergic neurons projecting to the spinal cord. ($\times 150$).

strongly labeled neuronal cell bodies (Figs. 1A and 3D) (cf. Kuypers et al., 1977, 1979; Bentivoglio et al., 1979b). The only tracer that gave a diffuse cellular staining in the present procedure was EB, which occurred throughout the cell bodies of the retrogradely labeled neurons (Fig. 2B). The granular storage of PI, TB, GB, DAPI and bisbenzimidazole (Fig. 1) resembles that of HRP, thus suggesting that these tracers may use the same retrograde transport mechanism as HRP. The EB transport appears to be different.

(B) Fluorescence colors and emission spectra

Both in albumin models and in labeled cells in tissue EB fluoresced red, PI orange-red, bisbenzimidazole whitish yellow, DAPI yellowish blue, and TB and GB had an ice-blue color. In Table 1 the corresponding emission maxima are listed together with the maxima of the formaldehyde-induced monoamine fluorophores. In the far-right column in Table 1 the relative fluorescence intensities of the native fluorescent tracers and monoamine fluorophores are listed. These intensities were recorded in protein models at maximum excitation wavelength.

DAPI was the most intensely fluorescent compound, its intensity being about 13-fold higher than that of the formaldehyde-induced catecholamine fluorophores. On equal weight basis (0.1 mg/ml) GB, TB and bisbenzimidazole had similar intensities, 5–6-fold higher than the catecholamine fluorophores. PI and EB had the weakest fluorescence, though still in the range of the catecholamine fluorophores. Since as little as 10^{-4} pg of the catecholamines are readily detectable in varicosities in the formaldehyde and glyoxylic acid histofluorescence methods (Jonsson, 1971; Björklund et al., 1975), it can be inferred that the limit of detection of the fluorescent tracers in granular storage should be in the order of 10^{-4} – 10^{-5} pg.

The catecholamine fluorophores have a yellow to yellow-green color with the maximum around 500 nm, and that of serotonin is yellow to brownish yellow with the maximum at about 530 nm in specimens processed according to the ALFA method. The fluorescence colors of DAPI and bisbenzimidazole are not clearly distinct from that of the catecholamine fluorophores, and the emission maxima are close to each other (490–500 nm for the tracers vs 490–520 nm for the catecholamines). By contrast, the reddish fluorescence of EB and PI, and the ice-blue fluorescence of TB and GB are easily distinguishable with the eye in the fluorescence microscope, and the emission spectra are sufficiently different from those of the monoamine fluorophores to allow a safe microspectrofluorometric distinction between the tracers and the monoamine fluorophores even when they are present in the same cells (Table 1 and Figs. 5 and 6).

(C) Excitation characteristics

All tracers had excitation maxima different from those of the catecholamine or serotonin fluorophores (Table 1). This makes it possible to distin-

TABLE 1

FLUORESCENCE EXCITATION AND EMISSION MAXIMA AND RELATIVE FLUORESCENCE INTENSITIES OF SOME FLUORESCENT RETROGRADE TRACERS AND BIOGENIC MONOAMINES IN PROTEIN MODELS AND IN NEUTRONAL CELL BODIES

Compound	Peak maxima in models ^a		Peak maxima in tissue ^b		Relative intensity in tissue (%)				Maximum a.c. intensity in models
	exc. max (nm)	em. max (nm)	exc. max. (nm)	em. max. (nm)	at 365 nm	at 405 nm	at 435 nm	at 500 nm	
Dopamine	(340) ^{d,e} ; 410	480	(340) ^e ; 415	490-520 ^f	30	100	70	0	100
Noradrenaline	(340) ^e ; 410	480	(340) ^e ; 415	490-520 ^f	30	100	70	0	100
Serotonin	365; 415	530	365; 410	520-540 ^f	95	100	90	0	30
DAPI	350	495	360	490-500	100	60	30	0	1300
Granular Blue	345	425	355	425	100	40	25	0	550
True Blue	360; (390)	410	365; (390)	420-430	100	20	1	0	500
Bisbenzimidide	350	480	360; (400)	glia = 480 neurons = 500	100	65	20	0	600
Propidium iodide	340 ^e ; > 500 g	600-610	340 ^e ; > 500 g	600-610	20	8	8	100	170
Evans Blue	c. 550 g	c. 750 g	c. 550 g	c. 750 g	0	0	0	100	80

^a Measured in dried protein droplets, prepared from 2% albumin solutions containing 0.1 mg/ml of the compound tested. The dried droplets were exposed to formaldehyde vapors for 1 h at +80°C. The specimens were carried on normal glass microscope slides.

^b Measured in non-labeled monoamine-containing neurons and in retrogradely labeled neuronal cell bodies (for bisbenzimidide also in glial nuclei) in brains prepared according to the ALFA method. The fluorescence intensities are expressed in percent of maximum intensity. All values have been connected for instrument factors, such as variable light flow and variable transmission at different wavelengths. When translating the intensity and peak maximum values to a normal fluorescence microscope with a mercury lamp source differences in illumination intensity and transmission through filters and optics in the particular instrumental set-up used must be taken into account.

^c Measured at maximum excitation wavelength and expressed with the intensity recorded from dopamine and noradrenaline as 100.

^d Figures in brackets denote second (lower) peak or shoulder in the spectral curve.

^e These peaks are displaced about 20 nm due to the high light absorption of the glass microscope slides below 350 nm. When measured in specimens carried on quartz slides the peak maximum was at 320 nm.

^f The maximum is concentration-dependent.

^g The value is approximate due to limitations in the instrumentation used.

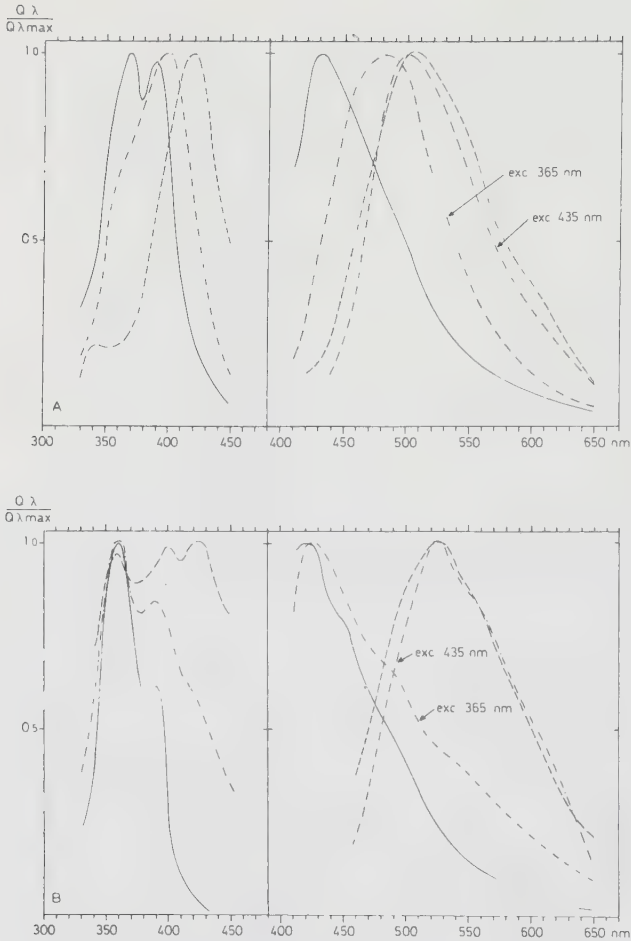


Fig. 5. A and B: fluorescence excitation spectra (left panels) and emission spectra (right hand panels) recorded in the brain stem after injection of PI into the cervical spinal cord. In A, (—) = A1 cell body containing TB alone; (----) = A1 cell body containing noradrenaline but no TB label; (- · - · -) = A1 cell body containing both noradrenaline and TB. In the latter case the emission curve recorded at 435 nm activation reveals the noradrenaline component in the mixture. In B, (—) = raphe magnus cell body containing TB alone; (----) = raphe magnus cell body containing serotonin but no TB; (- · - · -) = raphe magnus cell body containing both serotonin and TB. As in A, the emission recorded at 435 nm activation demonstrates in the latter case the serotonin content of the cell analysed.

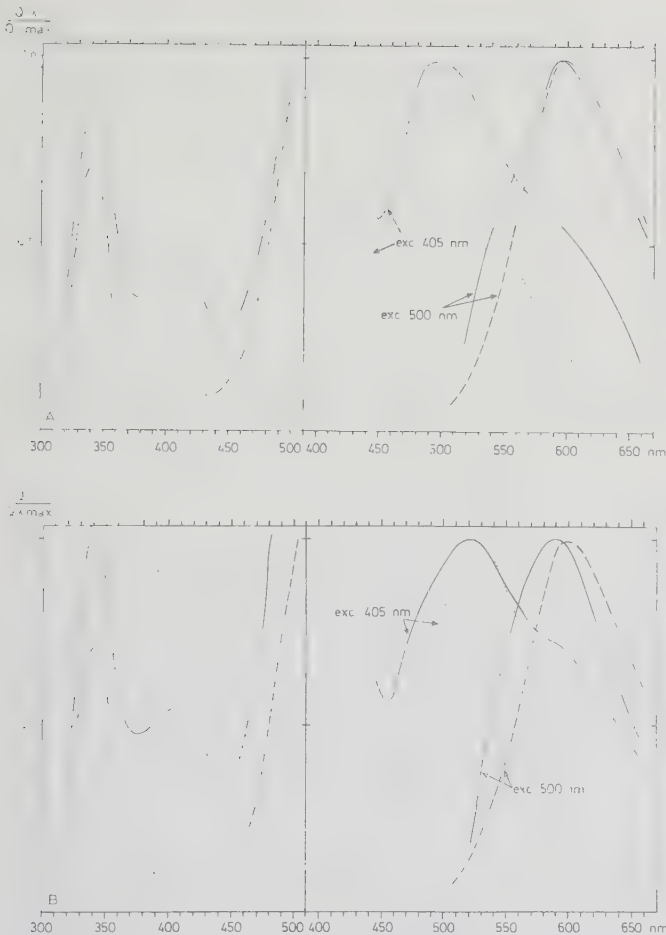


Fig. 6. A and B: fluorescence excitation spectra (left panels) and emission spectra (right hand panels) recorded in the brain stem after injection of PI into the cervical spinal cord. In A, (-----) = pontine reticular cell body containing PI alone; (.....) = locus coeruleus cell body containing noradrenaline but no PI label; (—) = locus coeruleus cell body containing both noradrenaline and PI. In the latter case the emission curves recorded at 405 and 500 nm activation reveal the noradrenaline and PI contents, respectively, of the cell analyzed. In B, (-----) = pontine reticular cell containing PI alone, (.....) = raphe pontis cell body containing serotonin but no PI; (—) = raphe pontis cell body containing both serotonin and PI. As in A, activation at 405 and 500 nm reveal the serotonin and the PI components, respectively, in the mixture.

guish all different tracers from the monoamine fluorophores, even those whose emission spectra (and hence fluorescence colors) are indistinguishable. In three cases, notably TB, PI and EB, the excitation characteristics proved, however, to be sufficiently different from those of the monoamine fluorophores to make possible reliable subjective distinction in the fluorescence microscope, without spectral recordings. EB and PI were maximally activated at 500–550 nm, i.e. at a wavelength range where the catecholamines and indoleamines (as well as the other tracers tested) yield no detectable fluorescence. Thus, EB-labeled cells (Fig. 2B) and PI-labeled cells (Fig. 3B, D) can selectively be visualized at 550 nm illumination, whereas the catecholamine or indoleamine content of the cells is visualized at 405 nm illumination (Figs. 2A and 3A, 3C). At this latter wavelength EB and PI are very weakly fluorescent (cf. Figs. 3 and 6).

TB, GB, DAPI and bisbenzimidazole yield maximum fluorescence in a wavelength range, 345–365 nm, where the monoamine fluorophores yield significant albeit suboptimal fluorescence (Table 1). Thus, when viewed under illumination from the 365 nm line of the mercury lamp both the monoamine content and the tracer will be visible. TB has, however, the favorable property of having practically no fluorescence at 435 nm (about 1% of the fluorescence recorded at 365 nm, see Table 1 and Fig. 5), while the intensity of the catecholamine and serotonin fluorophores are close to maximum at this wavelength. Thus, as illustrated in the photographs in Fig. 4, illumination of a section from a TB-labeled specimen at 435 nm will selectively visualize cells containing catecholamine (Fig. 4A) or serotonin (Fig. 4C), whereas the bright, ice-blue TB-fluorescent granules will appear in the labeled cells when the illumination is switched to 365 nm (Fig. 4B, D). Non-labeled monoamine-containing cells will remain with a weaker yellowish cytoplasmic fluorescence. GB, DAPI and bisbenzimidazole are clearly less well suited for this kind of subjective distinction since they yield stronger fluorescence than TB at 435 nm (20–30% of maximum, see Table 1).

The highly useful properties of the TB and PI labelings are illustrated in the spectral recordings in Figs. 5 and 6, where the left hand panels give the excitation spectra and the right hand panels the emission spectra recorded from labeled and non-labeled neuronal cell bodies in the brain stem after injections into the cervical spinal cord (3 days survival). The spectra recorded from non-monoaminergic cells labeled with TB or PI (solid lines) are widely different from the spectra recorded from non-labeled monoamine-containing cells (dashed lines) in both cases. In cells containing both a monoamine and the TB or PI tracer the excitation spectrum represents a summation of the tracer and the monoamine curves (dash-dotted line in left panels in Fig. 5, and dotted lines in left panels in Fig. 6). In case of TB-labeled monoamine-containing cells activation at 365 nm gives an emission curve that is intermediary between the TB and the catecholamine or serotonin curve, whereas activation at 435 nm gives a pure catecholamine or serotonin curve due to the very weak TB fluorescence at this wavelength (dash-dotted lines in right

hand panels in Fig. 5). With PI-labeled monoamine-containing cells, activation at 500 nm gives an emission curve that is close to that of pure PI, and activation at 405 nm yields emission curves where the catecholamine or serotonin peaks predominate (dotted lines in right hand panels in Fig. 6).

(D) Recommended procedure

Of the compounds tested EB, PI and TB are clearly the ones most ideally suited for identification of labeled cells in the presence of monoamine fluorescence in the fluorescence microscope. These compounds are sufficiently different from the monoamine fluorophores with respect to both fluorescence colors and excitation maxima to allow safe distinction in most cases of the tracer and the monoamine fluorophore without spectral recordings, also when they are present in the same cell. Moreover, the red-fluorescent tracers, EB or PI, in combination with the blue-fluorescent TB can be recommended for double-labeling studies on the collateral projections of monoamine-containing neurons.

Tissue preparation

While the intracellularly stored tracer substances are bound sufficiently well to resist a frozen-sectioning procedure similar to that commonly used for HRP tracing, the monoamines will diffuse out from the cells under such conditions. Thus, a preparative procedure compatible with monoamine visualization must be used. The ALFA method and the standard Falck-Hillarp formaldehyde method have great advantage in that the paraffin-embedded material allows convenient serial sectioning of large parts of the CNS, and in that it is easy to store, either en bloc or in the form of mounted or unmounted sections. The specimens can be stored in a freezer (-20°C) for many months without notable loss in quality.

Microscopy

For microscopy of *TB-injected specimens* the fluorescence microscope should be equipped with lamp filters for selective illumination with the 365 and the 435 nm lines of the mercury lamp. A 2 mm UGI filter (Schott; 75% transmission at 365 nm) and a Leitz AL 435 band interference filter (40% transmission at 435 nm) are quite appropriate. Zeiss 41 + 65 and 44 + 47, respectively, are recommended as secondary filters. The section is first viewed under 435 nm illumination, which shows the cytoplasmic catecholamine and serotonin fluorescence in the virtual absence of TB fluorescence. A photograph taken at this wave-length shows selectively the catecholamine- and serotonin-containing cell bodies (Fig. 4A, C). Switching to 365 nm illumination will add the bright, granular TB fluorescence to the picture, while the catecholamine and serotonin fluorescence is somewhat reduced. A photograph taken at this wavelength will thus show the monoamines plus

TB (Fig. 4B, D). A monoamine-containing cell body that is labeled will increase its fluorescence, become granular and assume a more bluish or violet color when switching from 435 to 365 nm illumination, while the fluorescence of a non-labeled monoamine cell body is somewhat reduced and remains agranular³. The fluorescence color is unchanged. A labeled non-monoaminergic cell has no cytoplasmic fluorescence at 435 nm and has the characteristic ice-blue, granular fluorescence at 365 nm. Ambiguous cases occur, particularly in strongly fluorescent catecholamine perikarya with weak TB labeling, where the strong amine fluorescence can mask the TB granules when viewed at 365 nm. In such cases, the TB labeling is, however, reliably detected by spectral analysis (see below).

In PI- or EB-injected specimens the microscope should be equipped with filters for illumination with the 405/435 nm lines and the 546/577 nm lines of the mercury lamp, respectively. The filter-mirror combinations B and N₂, respectively, of the Leitz Ploem-Opak illuminator are suitable for this purpose. Illumination of the section at 546/577 nm shows selectively the PI and EB labeled cell bodies (Figs. 2B and 3B, D). On switching to 405/435 nm illumination the orange-red PI fluorescence and the red EB fluorescence is greatly reduced and the yellow to yellow-green catecholamine and serotonin fluorescence are added to the picture (Figs. 2A and 3A, C). Weakly labeled cells can be difficult to distinguish from cells containing autofluorescent granular pigment. Positive identification of weakly labeled neurons is possible provided that non-injected control specimens have shown that they contain no interfering pigment. The monoaminergic cell bodies are often pigmented, however, and in such cases faint labeling can only be recorded through spectral recordings.

Spectral analysis

It is a highly attractive feature of the fluorescent retrograde tracers that they can be microspectrofluorometrically identified with high sensitivity in labeled cell bodies. In fact, a microspectrofluorometer is far more sensitive than the eye in picking up individual fluorophores in mixtures. When having three or four fluorescent markers present in the same sections — the catecholamines and indoleamines, plus one or two fluorescent tracers — spectral recordings should therefore be performed in all doubtful cases. It is of particular value when two or three fluorophores are present in the same cell in widely varying proportions. According to our experience the presence of small amounts of TB or PI in strongly fluorescent catecholamine or sero-

³ The reduction of the intensity of the noradrenaline and dopamine fluorescence is most reliably seen in specimens processed according to the Falck-Hillarp procedure. In aluminum-perfused specimens the acidity of the perfusion solution can, particularly in well-perfused areas, cause a partial acid-induced shift of the excitation spectrum, which makes the intensity of the catecholamine fluorophores higher at 365 nm than at 435 nm. The dashed curve in Fig. 5A and the dotted curve in Fig. 6A, recorded from aluminum-perfused specimens, show, however, that this shift often does not occur.

tonin cell bodies can reliably be picked up both in the excitation and emission spectra also in cases when subjective distinction of the tracer is difficult. The best identification is obtained if both emission and excitation spectra are recorded and if emission spectra are registered at, or near, the maximum excitation wavelength for each of the fluorophores present, such as done in Figs. 5 and 6.

The recording of excitation spectra is of particular importance for TB—catecholamine mixtures where the TB—catecholamine ratio is low. In such cases model experiments have shown that a two-step activation of the *catecholamine* fluorescence occurs when the *true blue* fluorophage is activated (a 'scintillation effect'). Thus, activation of TB at maximum excitation wavelength (350–360 nm) produces a fluorescence with maximum at about 410 nm, which in turn is absorbed by the relatively more abundant catecholamine fluorophores. In such cases the emission spectrum will look like a relatively pure catecholamine curve, despite that the excitation spectrum has the expected shape of a TB—catecholamine mixture.

DISCUSSION

The results show that all fluorescent retrograde tracers tested can be used in conjunction with monoamine fluorescence and that freeze-dried, paraffin-embedded material is excellent for this purpose. Bisbenzimidazole and DAPI clearly required microspectrofluorometric analysis of excitation spectra in order to be identified within fluorescent catecholamine-containing cell bodies. TB, GB, PI and EB differ sufficiently in color to be distinguishable from both catecholamine and indoleamine fluorophores with the eye in the fluorescence microscope. Of these, TB, PI and EB are clearly the ones most ideally suited for retrograde tracing in monoamine fluorescence histochemistry since they allow selective visualization of the monoamine fluorophores (at 405–435 nm illumination) in sections from labeled specimens. In addition TB and PI have, in the freeze-dried specimens, a characteristic granular localization, which, in well-labeled cells, makes the label stand out against the more diffusely distributed monoamine fluorescence. In fact, the different properties of PI and EB (red to orange fluorescence, activation at about 550 nm), on the one hand, and TB (ice-blue fluorescence, activation at 365 nm), on the other, make them ideal for double labeling studies in combination with the yellow-green and yellow to brownish-yellow fluorescence of the catecholamines and indoleamines, respectively. This combination thus makes possible the simultaneous use of four different fluorescent markers — two tracers and two transmitter-derived fluorophores — in one and the same section.

The fluorescent tracers have great practical advantages over HRP as retrograde tracers. The time-consuming reaction of individual sections is thus avoided. When using paraffin-embedding, as in the present procedure, specimens can conveniently be stored in a freezer for prolonged periods before

sectioning, and the entire section series as well as remaining parts of the brain can easily be saved and stored until the microscopic analysis is finished. The advantages of the fluorescent tracers are even more obvious when applied to fluorescence histochemistry. While HRP tracing in combination with monoamine fluorescence requires restaining and re-photography of pre-selected areas, the use of fluorescent tracers does not involve any extra steps beyond the procedure used for the monoamine histochemistry itself. The simultaneous visualization of the tracer together with the monoamine fluorophores makes the present technique highly convenient and ideal for screening of large areas of the CNS.

Many aspects and characteristics of fluorescent compounds as retrograde tracers have yet to be analyzed. Our yet preliminary observations indicate, for example, that several of the compounds cause significant necrosis at the site of injection, when used in the present concentrations, and that they (partly as a result of this) can exhibit quite extensive transport in damaged axons-of-passage. Moreover, the leakage of tracer from labeled neurons and axon bundles, which is evident for at least bisbenzimidazole, PI and TB may cause problems of transneuronal transfer after longer postinjection survival times. However, with such possible limitations in mind the fluorescent retrograde tracers should provide a new powerful tool for detailed studies of monoaminergic axonal projections and collateral arrangements. Moreover, with slight modifications the present approach should be equally well suited for transmitter-specific tracing based on immunohistochemistry or enzyme histochemistry.

ACKNOWLEDGEMENTS

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FURTHER STUDIES ON THE USE OF TRUE BLUE AS A
RETROGRADE FLUORESCENT TRACER IN COMBINATION
WITH MONOAMINE HISTOCHEMISTRY

G. Skagerberg, A. Björklund and O. Lindvall

ABSTRACT

Some basic methodological aspects on the use of the retrograde fluorescent tracer True Blue (TB) was studied in freeze-dried material in the rat CNS. On basis of fluorescence morphology and intensity at different filter settings it was possible to objectively delineate three distinct zones of the TB injection site, and define degrees of retrograde labelling from the different zones. Correlative studies of injection sites in subportions of the spinal cord and retrograde labelling in cell bodies of defined nuclei in the brainstem and cortex showed that effective uptake occurred only from the central area of the injection (zone I), while the uptake from the less intensely fluorescent zone II was variable and could only be documented for systems terminating within this zone. Time-course studies revealed that the size of the injection site and the resulting retrograde labelling is stable up to at least two months after injection and that relatively long survival time is often needed for optimal labelling. For practical purposes a bulk transport velocity of 20 mm/day can be used for estimating the survival time required for reasonable retrograde labelling. The accumulation of TB did not interfere with the visualization of monoamines in the same neurons, and the tracer was never seen to be anterogradely or transcellularly transported.

Key words: Fluorescent retrograde tracers, retrograde transport, True Blue.

INTRODUCTION

The use of fluorescent dyes for retrograde tracing of neuronal connections was pioneered by Kuypers and collaborators in the late seventies (Kuypers et al. 1977, 1979; Bentivoglio et al. 1979, 1980a,b). One major advantage with this methodology is the possibility to use simultaneously two different tracers, which allows for anatomical studies on the collateralization patterns of individual neurons (see e.g. van der Kooy et al. 1978; Kuypers et al. 1980). The fluorescent retrograde tracers can also be used in conjunction with fluorescence histochemical methods for transmitter detection (see Hökfelt et al. 1983, for review) and in this way information can be gained not only about the projection of an individual neuron but also of its neurotransmitter content. This approach has been profitably used in studies where fluorescent tracers have been combined either with immunocytochemistry for transmitter-related compounds (van der Koy and Steinbusch 1980; Sawchenko & Swanson 1981; Hökfelt et al. 1981) or with aldehyde-induced monoamine histofluorescence (Björklund and Skagerberg 1979a,b; Nagai et al. 1981).

We have found that the fluorescent retrograde tracer True Blue (TB) is particularly well suited for combination with aldehyde-induced monoamine histofluorescence (Björklund and Skagerberg 1979a; Hökfelt et al. 1983). But despite that TB has been in use as retrograde tracer for several years, there is still a number of basic methodological aspects, of importance for the proper interpretation of experimental data, that remain to be studied more systematically. For this reason, we have made some experiments especially designed to provide more information on the behaviour of this tracer in freeze-dried material. The main objectives of these experiments were threefold: (1) to define the area of efficient tracer uptake around the injection site; (2) to investigate how fast the tracer is transported and how long survival time is needed for transport over long distances; and (3) to check whether the retrograde accumulation of TB interferes with the visualization of monoamines in monoaminergic cell bodies. Though it is important to note that all the results reported here were obtained in freeze-dried tissue from rat brain and spinal cord, it seems likely that the principal findings apply also to other species and techniques of tissue-processing.

MATERIALS AND METHODS

Animals. Throughout the study female Sprague-Dawley rats were used, usually weighing 180-200 g at the time of tracer injection.

Tracer injections. A suspension of 2-3% (w/v) TB in distilled water was used. It was stored in a freezer (-20°C), thawed and sonicated for about one minute before use. The tracer was injected, under ketamin xylazine anaesthesia (50 and 10 mg/kg, respectively, i.p.) either directly through the cannula of a 1 μl Hamilton syringe or through a glass capillary (outer diameter approximately 100 μm) attached to the cannula. When utilizing a glass capillary for injection, the whole system was filled with paraffin oil in order to minimize the problem with compressible air. The tracers were in most cases delivered slowly (about 0.01 $\mu\text{l}/\text{minute}$) by means of a mechanical microdrive and the capillary or cannula left in place for at least one minute before being slowly retracted.

Injectons into the brain were performed stereotactically, the rats being mounted in a Kopf stereotaxic apparatus. Two different approaches were used for spinal cord injections. For cervical injections the animals were conventionally mounted in a stereotaxic frame, whereafter the tail was gently stretched, serving both to suspend the animal, so that respiratory movements did not cause any observable motion of the vertebral column, and at the same time to increase the intervertebral space. The neck muscles were separated from the underlying vertebrae and the intervertebral ligament cut through, exposing the dorsal surface of the cervical cord. For injections at lower spinal levels a laminectomy approach was used: The animals were loosely secured onto a tabletop by adhesive tape over the extremities. After incision through the shaved skin the dorsal tips of the spinal processes were localized and longitudinal cuts were made along the processes until three or four vertebrae were exposed. The connective tissue between two adjacent vertebrae was cut through so that the most rostral could be elevated enough to expose the spinal canal, the arcs of this vertebra was cut, the lamina folded forward and temporarily fixed in this position. The dura was then cut with the tip of a fine cannula and in some cases the position of the cord was secured by matressing the space between the dura and the bone with small pieces of gel-foam.

Perfusion: After varying survival times (see below) the animals were deeply anaesthetized with chloral hydrate (500 mg/kg, i.p.) and perfused with 100 ml ice-cold Tyrode's buffer followed by 250 ml ice-cold aluminum-formaldehyde (ALFA) solution (Lorén et al. 1980) with pH adjusted to 4.7. Pieces of the brain and spinal cord were then removed, rapidly frozen to the temperature of liquid nitrogen, and freeze-dried for five days, after which the tissue was exposed to formaldehyde vapour and embedded in paraffin in vacuo. Sections were finally cut at 15 μm , mounted in Entel-

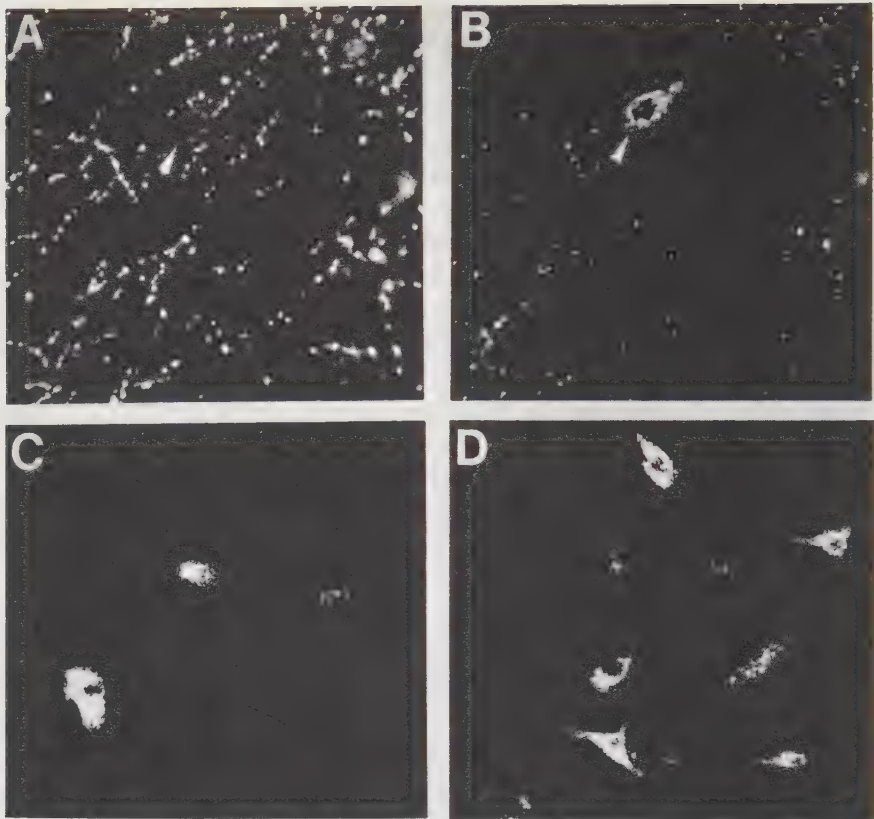


Figure 1. Some examples of the appearance of TB-labelled neurons in freeze-dried material. A and B show the same field of view at 435 and 365 nm excitation, respectively. At 435 nm weakly autofluorescent cellbodies are seen among the monoaminergic fibres, when the excitation light is changed to 365 nm both the monoamine- and the auto-fluorescence decrease in intensity while a TB-labelled cell body becomes quite conspicuous (arrowhead). A more weakly labelled neuron is seen in the left corner (small arrow). In C and D cells with moderate or strong labelling are seen. In D some labelling in proximal processes are also apparent. Magnification: 230X (A,B), 196X (C,D).

lan and cover slipped. For further details, see Björklund (1983) and Hökfelt et al. (1983).

Fluorescence microscopy: For visualization of TB and/or monoamines in the same section a Zeiss junior microscope for transmitted light was used. A HBO 200 lamp was used as light source and Schott UG 1 or AI 435 as primary filters for excitation at 365 and 435 nm, respectively. At 435 nm excitation Zeiss 47 was always used as barrier filter and with excitation at 365 nm Zeiss 44 or 47 or no secondary filter was used. In the following, the filter setting will be indicated in brackets, e.g. (365+44), where the first number indicates the excitation wave length and the second the secondary barrier filter.

Cell counts were made in defined regions (see below), counting cellular profiles larger than $10 \times 10 \mu\text{m}$ and correcting for double-counts by applying the formula of Abercrombie (Abercrombie 1946).

RESULTS

1. Appearance of retrogradely labelled cells

In freeze-dried material cells which contain retrogradely transported TB are usually easily identified in the (365+0) filter setting, in which the tracer is observed as ice-blue granules in the soma and proximal part of the processes (Fig. 1). The degree of TB labelling of an individual neuron can be classified as weak, moderate, strong, or very strong. The weak labelling is seen especially at short survival times and consists of a weak, dark-blue and sparse, granularity confined to the cell body and only visible using the (365+0) filter setting. Such weakly labelled cells may erroneously be discarded as containing natural pigment rather than TB tracer. However, this can be avoided by shifting to the (435+47) filter setting which will reveal the nature of the granules, since at this filter setting TB labelling disappears completely while the natural pigment is still visible (Fig. 1A). Moderate labelling (cf. Fig. 2A) refers to cells where most of the soma contains light-blue TB granules that are easily identified using the (365+0) or (365+44) filter settings and which to a lesser degree also are visible using the (365+47) combination. Strong labelling (cf. Fig. 2B) is easily seen also with the (365+47) combination and is very conspicuous at (365+0), where it is observed as fairly densely packed granules with a whitish-blue fluorescence. Very strong labelling is seen only under special circumstances in certain cell groups; it usually requires long survival times, large injections and/or close proximity to the injection site. Such labelling is seen as an intense whitish fluorescence with the (365+0) combination in which individual granules are hard to distinguish.

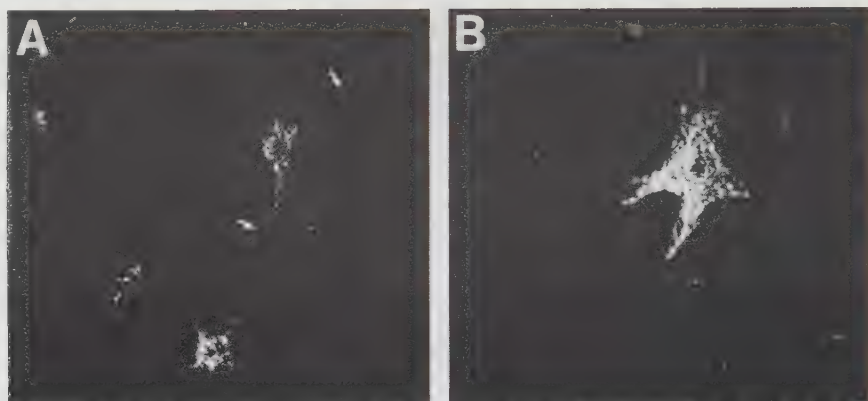


Figure 2. A: Two retrogradely labelled cells from one of which tracer filled processes can be followed for some distance (small arrows; X240). B: A larger and more strongly labelled cell in which the TB granules more densely are filling the proximal parts of some processes; x310.

Fluorescence fading of TB can be a problem with weakly labelled cells thus adding to the difficulty of photographically documenting the presence of weakly labelled neurons. By contrast TB fluorescence is notably stable in moderately and strongly labelled cells.

Retrograde labelling more peripherally in the processes is sometimes seen (Fig. 2), but the concentration of TB is always highest in the soma and the tracer appears exclusively located in cytoplasmic granules. This is in contrast to the appearance of TB when the tissue is processed by other techniques than freeze-drying. When using "wet" techniques (including rinsing in buffer and cryostat sectioning) the TB is generally reported to be more evenly distributed throughout the perikarya and proximal processes and exhibiting no or little granularity (Huisman 1983; Hökfelt et al. 1983).

The appearance and identification of retrogradely labelled monoaminergic neurons have been illustrated and discussed elsewhere (Björklund and Skagerberg 1979a; Hökfelt et al. 1983; Skagerberg and Björklund 1984) and will here only briefly be commented upon. If monoaminergic neurons are first excited at 435 nm (435+47) they will exhibit their specific catecholamine- or indolamine-fluorescence. If the excitation light is then changed to 365 nm (365+47) monoaminergic cells which contain the tracer will increase in fluorescence intensity and also show an added granularity in the cytoplasm. For catecholamine-containing neurons both these criteria, i.e. increased fluorescence intensity and the appearance of cytoplasmic granules, are necessary conditions to be fulfilled if a neuron is to be judged as being retrogradely labelled. For serotonergic neurons we have, however, observed some cells which, though being retrogradely labelled, do not exhibit any granularity. The presence of TB in these cells will be indicated only by a shift in their emitted light towards the blue end of the spectrum when changing the filter setting from (435+47) to (365+0).

2. Injection sites

2.1 Fluorescence morphology and zones of tracer spread

By observing differences in morphology and fluorescence intensity three different zones of the injection site are possible to delineate on basis of general fluorescence morphology and extent at 435 and 365 nm excitation, as illustrated in Fig. 3. The central area of the injection site, zone I, exhibits a homogenous accumulation of TB which is very intensely fluorescent at 365 nm but also clearly seen at 435 nm. In zone I the normal cytoarchitecture is totally obliterated and completely replaced by the

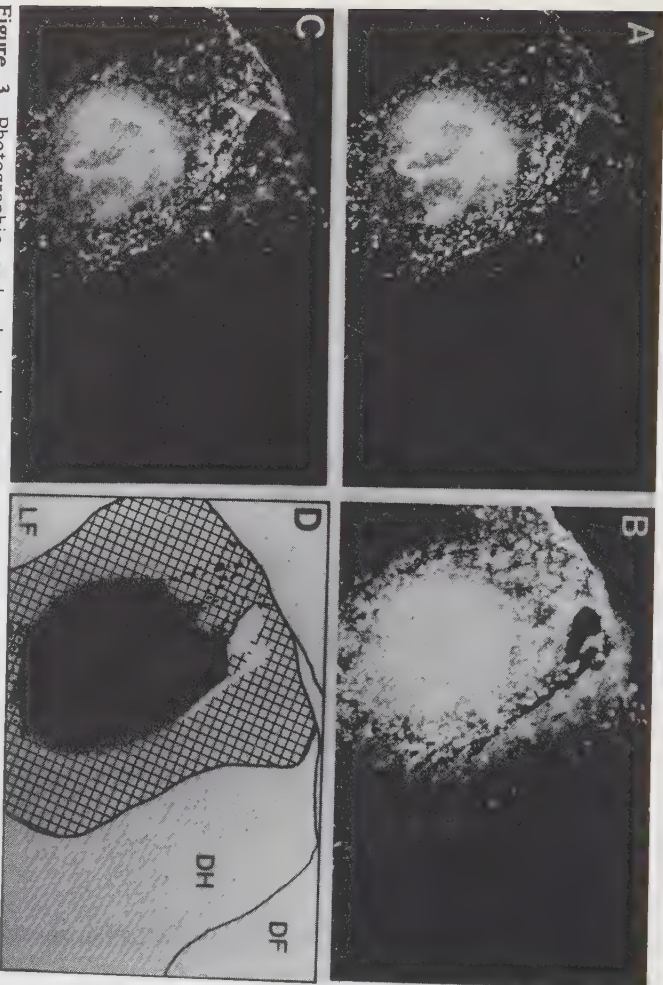


Figure 3. Photographic and schematic representation of a small injection site. $0.05 \mu\text{l}$ TB was injected into the lateral part of the dorsal horn and adjacent lateral funiculus. A: Excitation at 435 nm reveals the central area, zone I, of the injection site and also visualizes some slender monoaminergic fibres in the dorsal horn. B: Excitation at 365 nm adds the now fluorescent zone II but abolishes the monoamine fluorescence due to the short exposure time. C: Double-exposure using both 365 and 435 nm excitation still reveals most of zone II but without completely obliterating the background. D: Schematized representation of the injection site which delineates zone I (solid black) and zone II (hatched area). DF, dorsal funiculus; DH, dorsal horn; LF, lateral funiculus. X110.

amorphous, or in some cases granular, deposit of tracer. Zone I is surrounded by an area (zone II) which shows intense tracer fluorescence at 365 nm but no TB fluorescence at 435 nm excitation. In zone II, normal tissue is preserved but seems completely filled with tracer. Although zone II usually occupies a fairly large area peripheral to zone I, it is occasionally absent. In such cases zone I is bordered directly by the external area, zone III, in which the tracer is exclusively intracellular, the cell bodies sometimes being very strongly labelled.

The border between zones I and II is usually easy to define within about 50 μm . Sometimes empty vacuoles are seen along this border though such spots of apparent tissue resorption may also be seen within the central zone. At this border there is often also some accumulation of fluorescent pigment probably of hematogenous origin which, however, by virtue of its yellow-brown emission colour is easy to differentiate from TB. Zone II more gradually merges with zone III but the border can usually be determined within 100 μm . The width of zone III is very difficult to define but since no retrograde uptake seems to occur from this area (see section 2.3) it can be left aside. Fig. 3 illustrates an injection site in the spinal cord with zones I and II clearly distinguishable.

2.2 Changes in the appearance of the injection site with time

In an attempt to assess the effect of different survival times on the size and morphology of the injection site, a group of six animals were injected in one session using the same glass capillary and injection parameters (0.1 μl TB was injected over one minute into the medial hypothalamus, the capillary left in place for another five minutes and slowly retracted during one minute). The rats were sacrificed at different time-points from 4 to 70 days after the injection, and the size and morphology of the injection sites were evaluated. In Fig. 4 the mean diameter of zones I and II are plotted against the corresponding survival time. As can be seen, there were no consistent changes in the sizes of the injection zones during the time-period studied, although the fluorescence of zone II seemed to exhibit some decrease in intensity at survival times longer than 4-5 weeks. This decrease in intensity made it increasingly difficult to determine the border between zones II and III. Concomitant with this intensity change a tendency to fragmentation of the fluorescent material in the central zone is sometimes observed. Thus, at longer survival times zone I may be seen to be made up by an accumulation of intensely fluorescent granules rather than the confluent and homogenous TB fluorescence seen at shorter survival times.

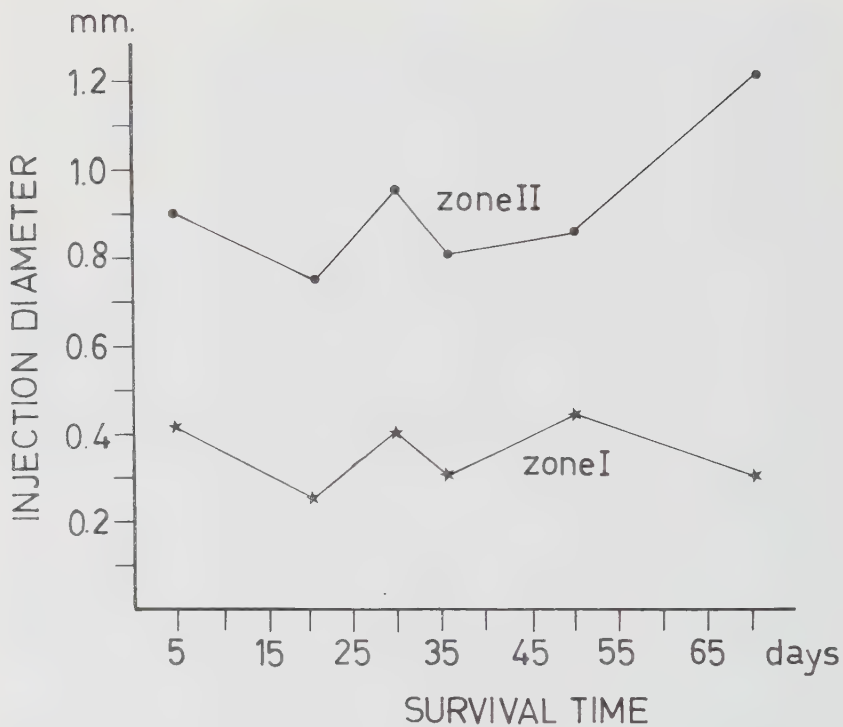


Figure 4. Representation of the mean diameter of zones I and II of the injection site after different survival times. (Injection volume 0.1 μ l).

2.3 Uptake of TB from different zones of the injection site

In order to determine to what extent uptake and subsequent retrograde cell body labelling occur from the three different zones of the TB injection site, a series of experiments were performed in which injections of different volumes of tracer were made into spinal segment C₅. The extent of zones I-III was then carefully mapped and the degree of retrograde labelling was recorded in some brain nuclei whose spinal projections and terminations are well known. The funicular trajectories and the terminal distributions of the systems taken advantage of in these experiments are schematically represented in Fig. 5A. (i) The corticospinal system (Cx) originates in the sensory-motor area of the neocortex and descends through the deep part of the dorsal funiculus and terminates exclusively in the dorsal horn (Brown 1971). (ii) The rubrospinal projection (Ru) originates in the red nucleus and projects through the dorsal part of the lateral funiculus to terminate in the intermediate zone and in the ventrolateral parts of the dorsal horn (Waldron and Gwyn 1971; Brown 1974). (iii) The spinal projection from nucleus laterodorsalis tegmentalis (TLD). We have not found any information in the literature concerning the localization at cervical levels of axons descending from this nucleus, but it seems reasonable to suppose that they have a distribution at C₅ similar to that seen at thoracic levels, i.e. laterally in the lateral funiculus (Loewy et al. 1979). These axons do not distribute any terminals at cervical levels but travers the cord to terminate at S₁ (Loewy et al. 1979). (iv) The raphe-spinal system (Ra) projects through the lateral and ventral funiculus to terminate in all areas of the grey matter (see Skagerberg and Björklund 1984).

The results from these experiments are summarized in Fig. 5B, which schematically represents some particularly illustrative injections. As can be seen, efficient retrograde labelling occurred from zone I irrespective of whether the central area of the injection involved terminal areas (such as the raphe projection in injection 2) or axons of passage (such as the TLD projection in injection 4). The retrograde labelling that could be ascribed to uptake from zone II seemed generally quite inefficient, both with respect to the number of retrogradely labelled cells and the degree of labelling, and it seemed critically dependent on the presence of terminals within the zone; apparently no uptake and subsequent neuronal labelling is possible through axons that pass through but do not terminate in zone II. This is exemplified by injection 1 which labelled some cortical neurons terminating within zone II but no TLD cells which send axons

Figure 5. Location of injection sites at C₅ and corresponding retrograde labelling in relation to some spinal projection systems.

In A the funicular trajectories and terminal distribution for spinal systems originating in the medullary raphe area (Ra), the laterodorsalis tegmentalis nucleus (TLD), in the sensory-motor cortex (Cx) and in the red nucleus (Rn). Though all of these systems are bilateral they have for the sake of clarity only been indicated on one side.

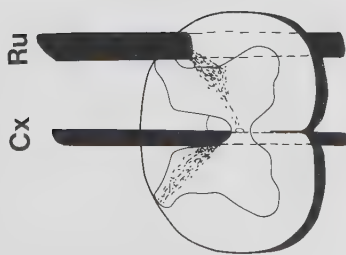
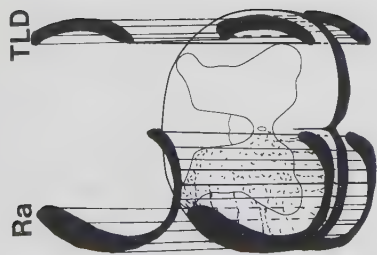
In B four injection sites with zones I and II indicated are shown together with the degree of retrograde labelling seen in the relevant areas (-, no retrogradely labelled cells; (+), occasional cells; +, intermediate number of TB cells; ++, many TB-cells).

B



Cx.	Ru.	Ra.	TLD.
+	++	++	-
++	+	+	-
-	(+)	-	-
(+)	++	++	++

A



of passage through this zone. No retrograde labelling was seen that could be ascribed to uptake from zone III.

2.4 Survival time required for retrograde labelling at different distances from the injection site

In order to determine how long survival times are needed for adequate retrograde labelling of cell bodies located at various distances from the injection site we injected large volumes (0.5–1.0 μ l) of TB into the lumbar enlargement of the spinal cord and evaluated the retrograde labelling along the neuraxis after different survival times (10,16,20,26,30,44,60, 93,122,140,160,170 and 190 hours post injection). The results are summarized in Fig. 6. No retrograde labelling outside the injection site could be seen at survival times shorter than 20 hours. At twenty hours weakly labelled cells could be seen within about 4 cm from the injection. During the next 20 hours such weakly labelled cells could be seen at successively more distant sites and was first observed in the cortex (i.e. approximately 12 cm away) after about 2 days. At this time moderately labelled cells could be seen up to high cervical levels while strong labelling was found within 1–2 cm from the injection. Moderately labelled cells were first seen in the cortex after about 60 hours at which time strong labelling was seen up to 3–4 cm from the injection. During the next few days strongly labelled cells were found at progressively more rostral positions but was not found in the cortex until after about one week's survival. Here it must be stressed that the labelling described hitherto refers to the strongest fluorescent cells seen; in most cases both strong and moderately labelled cells were seen side by side, weakly labelled cells were, however, not often seen after survival times exceeding four days. In other experiments with injections into the spinal cord, our observations indicate that the number of strongly labelled cells increases with prolonged survival times (up to 8 weeks) in some areas, and such long survival times also increase the incidence of very strong labelling. It is clear, though, that long survival time is not, per se, sufficient for the stronger types of labelling, which seems critically dependent on other factors; i.e. type of neuron, size of injection and distance from injection site. Thus, we have, for example, irrespective of survival time, never seen any very strongly labelled cortical neuron after injections into the cervical spinal cord. In contrast, injections at even greater distances from the cell bodies can produce very heavily labelled cells, e.g., in the nucleus ruber and the vestibular nuclei after lumbar injections, already at survival times of about 2 weeks.

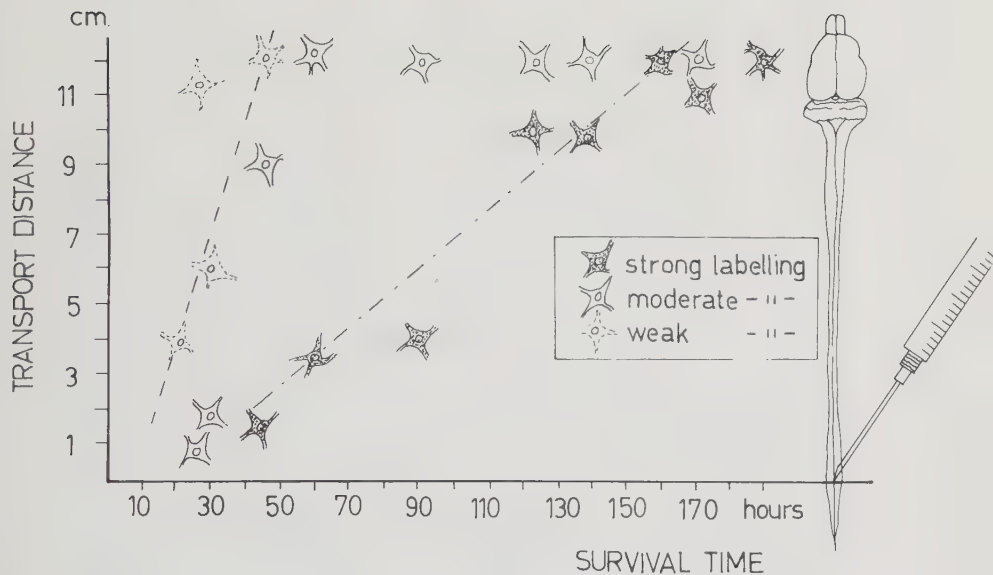


Figure 6. Relation between survival time and appearance of labelled cells at various distances from the lumbar injection site. Each symbol marks the maximal distance at which cells with the indicated degree of labelling could be found at each individual time-point. No retrogradely labelled cells were seen until after 18 hours' survival when weakly labelled cells could be seen up to mid-thoracic levels. Strongly labelled cells were not seen until after about 2 days' survival and then only at closely situated spinal levels, though at the same time-point, moderately labelled cells could be seen all the way up to cervical spinal levels, and weakly labelled cells were found in the cortex. Strong labelling was not reproducibly seen in the cortex until after about one week's survival. Longer survival times may in some systems further increase labelling, but this is then critically dependent on other factors such as injection volume and site of injection. The dashed line marks the calculated maximum transport velocity, and the dashed-dotted line the slow bulk transport velocity, as defined in the text.

2.5 Estimation of rates of intraneuronal transport

The present data allow for an approximate estimation of the rates by which TB is retrogradely transported. From Fig. 6 it seems clear that there are at least two different rates by which TB is transported in the present systems. The maximum transport velocity is defined by the first appearance of detectable amounts of the tracer at different rostral levels. Since weakly labelled cells were found at mid-thoracic levels at about 20 hours after injection, and at cortical levels (about 8 cm further away) about 20 hours later, this velocity can be calculated to be in the range of 100 mm/day (see dashed line in Fig. 6). The slow bulk transport velocity is defined by the first appearance of strongly labelled cells at different rostral levels. Such cells were found at mid-thoracic levels after about 60 hours, and at cortical levels about 100 hours later, giving a transport velocity in the range of 20 mm/day (see dashed-dotted line in Fig. 6).

3. Effect of retrograde labelling on the monoamine fluorescence in monoaminergic neurons

In an attempt to determine to what extent retrograde transport and subsequent accumulation of TB might interfere with the visualization of monoamines in cell bodies, the number of dopaminergic (DA), noradren-ergic (NA) and serotonergic (5HT) cell bodies in three different regions were counted in normal animals and in animals which had received large tracer injections into the dorsolateral part of the spinal cord. DA neurons were counted in the A11 area in the caudal and dorsal hypothalamus (see Skagerberg and Lindvall 1984). NA cell bodies were counted in the A5 cell group (Dahlström and Fuxe 1964), which is situated just medial to the root fibres of the facial nerve and just dorsal to the superior olive. The delineation towards cells of the subcoeruleus group were made by excluding cells that were located more than about 200 μm dorsal to the dorsal surface of the superior olive. 5HT cells were counted in the medullary arcuate cell group ventral and ventrolateral to the pyramidal tract, as defined by Skagerberg and Björklund (1984). As shown in Fig. 7, very similar numbers of cells exhibiting monoamine fluorescence were seen in both normal and tracer-injected animals. Also the quality of the monoamine fluorescence seemed similar in both groups.

4. Anterograde transport, trans-cellular labelling, and leakage from labelled cells

Throughout our studies we have never been able to document any anterograde transport of TB from cell bodies at the injection site to distan-

no. of MA-cells

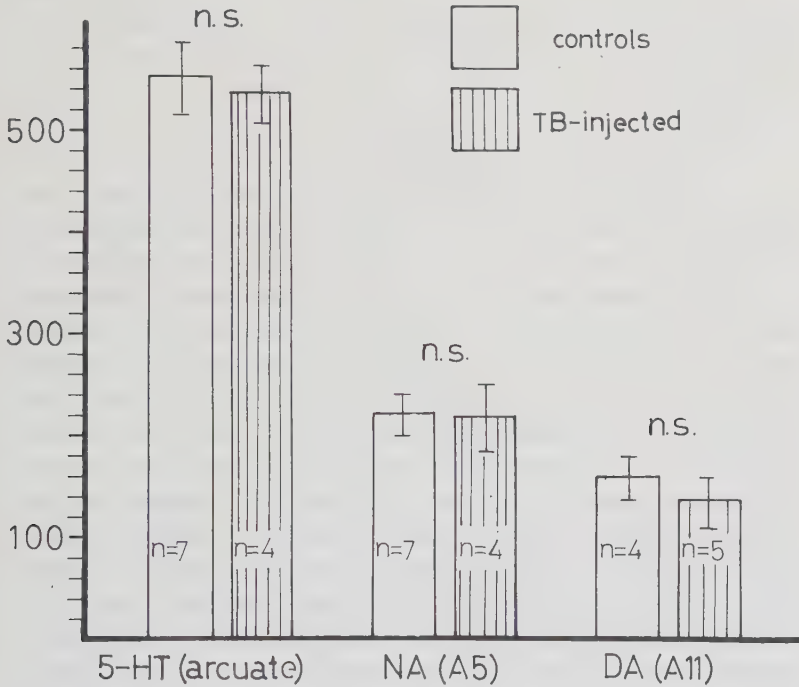


Figure 7. Mean numbers of cells ($\pm$ S.D) exhibiting monoamine fluorescence in three different brain stem regions. No significant difference between animals which had received tracer injections and normal animals was found ($p > 0.05$, Student's t -test). 5-HT containing cells were counted in rats pretreated with the MAO inhibitor pargyline (200 mg/kg, i.p., 3 hrs before killing).

tly located terminals. In keeping with this we have never observed any labelled fibre-bundles outside the injection site.

Cases with many very strongly TB labelled cells (i.e. labelled motoneurons in the spinal cord after injections confined to the spinal roots) were especially checked for signs of trans-cellular labelling. Although such cells were occasionally surrounded by a faint fluorescent halo no evidence for trans-cellular uptake and subsequent retrograde labelling was ever found. Also glial labelling was never found outside the injection site.

DISCUSSION

It is of vital importance for any tracing technique that the borders of the injection site can be rather precisely defined and the degree of tracer uptake from different areas of the injection site is known. On basis of differences in tissue morphology and fluorescence intensity we have divided the TB injection site into three different zones (I-III). Our data indicate that significant retrograde transport of the tracer only occurs from zones I and II. The delineations of zones I and II of the TB injection site, which probably correspond to the inner and outer zone described by Huisman et al. (1981), are easy to distinguish by changing the filter settings. The distinction between the two zones is important to make since it appears that efficient uptake and subsequent retrograde labelling through both axons of passage and terminals occurs only from zone I while the uptake from zone II is quite variable and seems to occur only through axon terminals. Thus, while retrograde labelling from zone II indicates the presence of axonal terminals, the absence of labelling from this zone does not provide any information. These results are in agreement with studies using other tracers, which have pointed to a differential uptake through terminals and axons of passage (La Vail et al. 1974; Nauta et al. 1974; Huisman 1983). It has been proposed that actual lesioning of axons of passage are of importance for retrograde labelling through these (Oldfield and McLachlan 1977; Condé and Condé 1979; Huisman et al. 1981). This may mean that in zone I TB is taken up not only by terminals but also by fibres-of-passage because the concentration of TB is sufficient to cause damage, or possibly only reversible injury to axons within this central zone, while in zone II the concentration of TB is sufficient to give some terminal uptake, but not enough to cause axonal injury. Sawchenko and Swanson (1981) were of the opinion that the uptake of TB by fibres-of-passage, which occurred from the injection site, took place in uninjured axons. Nevertheless, although the axons in this area

are not directly damaged by the injection needle or capillary, it remains difficult to tell from light microscopic sections whether the axons are indeed uninjured in the central zone of TB spread. In fact, this central zone, where the concentration of TB is highest, shows general signs of tissue disintegration which may suggest that TB has some general cytotoxic properties.

The long-lasting retention and extreme stability of the TB injection site, and the observation that the cellular labelling may actually increase during the first several weeks post injection, is in marked contrast to the results obtained with horseradish-peroxidase (HRP). This tracer is reported to rapidly diffuse from the injection site and disappear from the labelled cells within 3-4 days (La Vail and La Vail 1974; Warret et al. 1981). As a result the size of the HRP injections changes markedly within the first days after injection, which creates problems for the exact delineation of the zone of efficient uptake in HRP tracing studies (ref. Vanegas et al. 1978). The stability in the size of the TB injection site demonstrated in the present study shows that TB has clear advantages in this respect.

The long-lasting retention of the cellular TB labelling has also several methodological advantages. First, this phenomenon means that the retrogradely transported tracer can accumulate over an extended time, resulting in a progressively more sensitive visualization of labelled neurons. This may be of particular significance for the demonstration of neurons with only sparse projection to the injected area. Secondly, the long-lasting retention of TB in the labelled cell bodies provides interesting experimental possibilities for studies of development, regeneration and transplantation of neurons, when the cells can be labelled with two different fluorescent tracers with several weeks or even months interval. This possibility has already been profited from developmental studies on cortical connections (Innocenti 1981; Stanfield et al. 1982).

With regard to the survival time that is required for optimal labelling over long distances the results reported here are in agreement with earlier observations which indicated that several days or even weeks were needed for good labelling (Sawchenko and Swanson 1981; Hökfelt et al. 1983; Huisman 1983). We could distinguish at least two different rates of transport for TB probably occurring through separate mechanisms. The fast transport has a velocity of about 100 mm/day but a limited capacity. This velocity fits well with other estimations of the rate of centripetal movement in test retrograde transport systems (see Grafstein and Forman 1980 for review). In case of the slower bulk transport system the calculated

value of about 20 mm/day should be regarded as a guide for estimating the survival time needed for appropriate labelling rather than an exact determination of the intra-axonal rate of transport. Obviously, the neuronal labelling seen in a cell body at any time point after it has shown the first signs of labelling represents an accumulation of tracer carried by the fast transport in addition to what may have been carried by the slower transport. Considering this our calculations have probably overestimated the velocity of the slow transport, which thus might correspond to rate of the slow retrograde transport (3-6 mm/day) demonstrated by Fink and Gainer (1980).

From the present data it is possible to make recommendations on suitable postinjection survival times for studies with TB in the rat CNS. Then, good labelling of structures within 3-4 cm from the injection site requires at least 2 1/2 days survival, and good labelling over maximal distances (e.g. from spinal cord to cortex) requires at least 1 week survival. For several types of systems it is, however, favourable to use even longer survival times. In this context, it is interesting to point out the delay that occurred before any retrograde transport seemed to commence. This delay can be estimated to be in the order of 10-15 hours. When calculating the time for sufficient retrograde labelling, this time period thus has to be added.

The survival time is, however, but one of several factors of major importance for the degree of retrograde neuronal labelling. For example, the amount of HRP detectable in the cell body is, at least in some systems, clearly correlated with the functional activity of the neuron (Eisenman and Azmitia 1981; Theodosis 1982; Peyronnad and Charron). There is also considerable evidence that the degree of retrograde labelling in the cell body is directly related to how large a fraction of the total terminal area of the neuron is present at the injection site (Jones 1975; Huisman et al. 1983). From our studies on the diencephalo-spinal DA system it even seems that in some highly collateralized systems, the parental cell bodies cannot be retrogradely labelled by TB injections confined to small areas of the terminal field. Instead, cell body labelling in this system requires tracer deposition in the area of the main axons (Skagerberg and Lindvall 1984).

The cell counts performed to assess any possible adverse effects of tracer-accumulation on the visualization of monoamines in monoaminergic neurons were prompted by our somewhat unexpected finding of large numbers of TB-labelled non-monoaminergic projection neurons, within areas containing monoaminergic cell bodies, after injections (Skagerberg

et al. 1984; Skagerberg and Björklund 1984). Our findings of similar numbers of monoaminergic neurons in tracer-injected and in control animals seem, however, to rule out such an interference and thus strongly support the notion of non-monoaminergic projections from many areas known to contain monoaminergic cell bodies.

The absence of signs of anterograde transport or transcellular labelling is in keeping with most other studies which have addressed this problem utilizing TB in the rat CNS (Sawchenko and Swanson 1981; Aschoff and Holländer 1982; Huisman 1983). Accordingly, the phenomena of this kind sometimes seen in the primary visual system (Weidner et al. 1983) are not typical when using TB in other systems in the rat CNS.

In conclusion, the present data further emphasizes the valuable and in some respects unique features of TB as a retrograde neuroanatomical tracer.

(i) Due to the extreme retention and stability of the TB at the injection site, the size of the injection changes very little even over extended postinjection periods, and the area of effective uptake is well definable in the fluorescence microscope.

(ii) The transport characteristics of TB are well defined: it appears to be transported exclusively in the retrograde direction (from both terminals and axons) and since the transport velocities are known it is possible to calculate suitable survival times. There is no evidence of secondary leakage or transcellular transport from the primarily labelled neurons.

(iii) The long retention to TB in labelled cells both increases the sensitivity of the tracer and makes it useful for special experimental purposes, e.g. during development and regeneration.

(iv) The spectral characteristics of TB makes it ideal to combine with both aldehyde histofluorescence and immunofluorescence using FITC-labelled antibodies. Furthermore, TB is apparently not harmful to the labelled neurons and does not interfere with the visualization of their intraneuronal monoamines.

Finally, it should be noted that the characteristics of TB dealt with in this paper are not generally applicable to fluorescent retrograde tracers as a group. It is clear from previous studies (e.g. Aschoff et al. 1982; Huisman 1983) that the individual fluorescent tracer compounds in use differ markedly in their features, such as uptake characteristics, transport velocity, retention, leakage and transcellular transfer, and properties of intracellular storage.

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EVIDENCE FOR A MAJOR SPINAL CORD PROJECTION FROM
THE DIENCEPHALIC A11 DOPAMINE CELL GROUP IN THE
RAT USING TRANSMITTER-SPECIFIC FLUORESCENT
RETROGRADE TRACING

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Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing

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The diencephalic A11 catecholamine (CA) cell group is known to be a major source of fibres running in the periventricular CA-containing projection system^{13, 15}. This fibre system is a component of the dorsal longitudinal fasciculus of Schütz, which is a bidirectional tract interconnecting the lower brain stem and spinal cord with the periaqueductal grey and hypothalamic and thalamic areas. Although the periventricular CA fibre system can be traced fluorescence-histochemically, at least down to the medulla oblongata, there is so far no evidence for any descending projections of the A11 neurones. However, recent horseradish peroxidase (HRP) studies on the direct diencephalo-spinal projections have demonstrated retrogradely labelled neurones in the area of the A11 CA cell group after HRP injections into the spinal cord^{8, 12, 17}, thus raising the possibility that the periventricular CA system indeed may possess descending projections innervating the spinal cord. In the present study we have investigated this possibility using the recently introduced fluorescent retrograde tracers^{1, 11} in conjunction with monoamine fluorescence histochemistry⁵. The results show that the neurones in the A11 area projecting to the spinal cord are both catecholaminergic and non-catecholaminergic, and that the vast majority of the CA-containing neurones in the A11 cell group innervate the spinal cord. While this study was in progress an HRP study by Blessing and Chalmers⁶ appeared, reporting similar findings for CA neurones in the dorsal hypothalamus in the rabbit.

Two different fluorescent tracers were used: trans-1,2-bis (5-amidino-2-benzofuranyl) ethylene-2 HCl (True Blue, kindly supplied by Dr. O. Dann, Erlangen, G.F.R.), and propidium iodide (Sigma). True Blue (TB)¹ was used in a 5% (w/v) solution in distilled water in 8 rats and propidium iodide (PI)¹¹ as a 0.75% suspension in distilled water in 4 rats. The injections (0.5–1.0 μ l) were made bilaterally into the cervical spinal cord in female Sprague–Dawley rats (180–200 g body weight). After 3 or 7 days survival the rats were freeze-dried and processed by formaldehyde fluorescence histochemistry^{3, 16} for concomitant visualization of monoamines and the fluorescent tracers, as described in detail elsewhere⁵. Serial paraffin sections were made of the injection site and the meso-diencephalic region, and every other section was mounted in Entellan (Merck, Darmstadt, G.F.R.) for fluorescence microscopy. The TB injected specimens

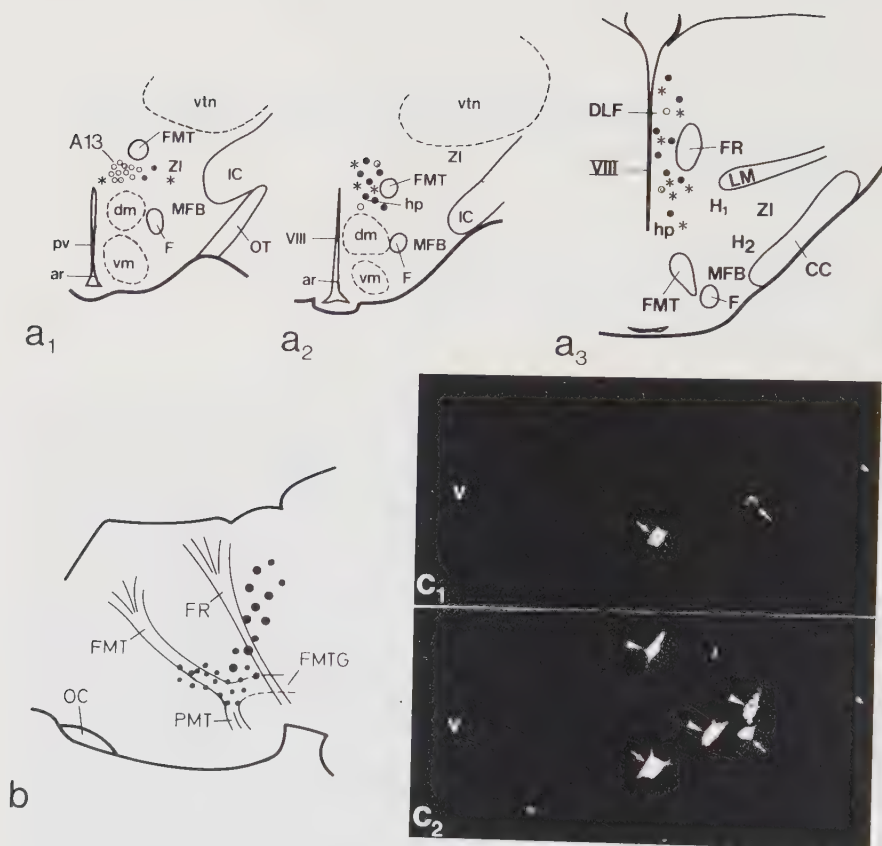


Fig. 1. a₁-a₃: schematic representation of the distribution of labeled CA-containing cell bodies (filled circles), non-labelled CA-containing cell bodies (open circles) and of labeled cell bodies lacking CA fluorescence (asterisks) in the A11-A13 area after injection of fluorescent retrograde tracer into the cervical spinal cord. b: distribution of retrogradely labeled CA-containing neurones in the diencephalon in a lateral projection (modified from ref. 4). c₁ and c₂: fluorescence photomicrographs from the posterior hypothalamus (level a₃) after injection of TB into the cervical spinal cord (3 days survival). c₁ shows selectively the CA fluorescence of two A11 cell bodies (arrows) at 435 nm illumination. c₂ shows the TB-labelled cell bodies in the same section field at 365 nm illumination. The ice-blue, granular TB fluorescence is now present in the two CA-containing cells, and in addition there occur 3 TB-labelled cell bodies (arrow heads) which contain no CA fluorescence. Abbreviations: ar, arcuate nucleus; CC, crus cerebri; DLF, dorsal longitudinal fasciculus; dm, dorsomedial nucleus; F, fornix; FMT, fasciculus mammillo-thalamicus; FMTG, fasciculus mammillo-tegmentalis; FR, fasciculus retroflexus; hp, posterior hypothalamic area; IC, internal capsule; LM, medial lemniscus; MFB, medial forebrain bundle; OC, optic chiasm; OT, optic tract; PMT, principal mammillary tract; pv, periventricular nucleus; V and VIII, third ventricle; vm, ventromedial nucleus; vtn, ventral thalamic nuclear complex; ZI, zona incerta.

were studied under illumination at 365 nm (Schott UGI lamp filter) and 435 nm (Leitz AL 435 band interference filter), and the PI injected specimens under illumination at 405/435 nm (Schott BG 12 filter) and at 546/577 nm (Leitz filter-mirror combination N₂), following the procedure described by Björklund and Skagerberg⁵. Microspectrofluorometric analysis was performed with a Leitz microspectrofluorometer, and the spectra were corrected for instrument factors³.

Consistent with previous HRP studies^{8,12,17} TB- and PI-labelled perikarya were found throughout the area of the A11 cell group, i.e. in the periventricular grey of the caudal thalamus (dorsal, medial and ventral to the fasciculus retroflexus), in the posterior and dorsal periventricular hypothalamus and in the medial zona incerta (along the medial and ventral aspects of the mamillothalamic tract), as summarized in Fig. 1a and b. A majority of the labelled cell bodies in these areas was CA-containing, as evidenced by the simultaneous occurrence of CA fluorescence and fluorescent tracer in about 1/2–3/4 of the labelled cell bodies. Moreover, the vast majority of the CA-containing A11 cells were found to be labelled. In Fig. 1, a₁–a₃, cells containing both CA fluorescence and fluorescent tracer are represented by filled circles, cells containing CA fluorescence but no tracer by open circles, and labelled cells with no CA fluorescence by asterisks. With the method employed the yellowish cytoplasmic CA fluorescence is selectively visualized in the fluorescence microscope at 435 nm illumination (Fig. 1, c₁). In the TB-injected specimens a shift of the illumination to 365 nm adds the ice-blue, granular TB fluorescence to the picture while cell bodies containing CA but no tracer become more weakly fluorescent (Fig. 1, c₂). In the PI-injected specimens a shift of the illumination from 435 nm to 546/577 nm visualizes the orange-red, granular PI fluorescence in the retrogradely labelled cells while the CA fluorescence completely disappears⁵. The presence of TB and PI label in the CA-containing cell bodies was further substantiated microspectrofluorometrically in specimens injected with TB or PI into the cervical spinal cord. In Fig. 2 the dotted lines show the excitation (left) and emission (right) spectra of a TB-labelled non-catecholaminergic cell body in the A11 area. These curves are indistinguishable from those of pure TB⁵. The dashed lines give the spectra of non-labelled CA fluorescent cell body in the ventral tegmental area. The spectra of an A11 cell body containing both TB-label and CA fluorescence is drawn in solid lines. These curves represent the combination of the TB and CA curves. The CA content of this cell is, moreover, revealed by the emission curve registered at 435 nm excitation.

The A11 neurones were the only diencephalic CA-containing cell bodies labelled by the present intraspinal tracer injections. In the area of the A13 cell group in the medial zona incerta (see a₁ in Fig. 1) the small, rounded and weakly-fluorescent cells of the A13 cell group proper were unlabelled, whereas the larger, angular and more strongly fluorescent cells in this area were clearly labelled. These latter cells are on morphological grounds referred to the A11 cell group⁴.

It is well established that the A11 cell system is primarily dopaminergic^{4,10}. Microspectrofluorometric recordings have indicated the presence of some noradrenaline-containing cells as well⁴, but this observation has so far not been confirmed with dopamine- β -hydroxylase immunocytochemistry^{9,10,19}. Thus, while it is clear that a

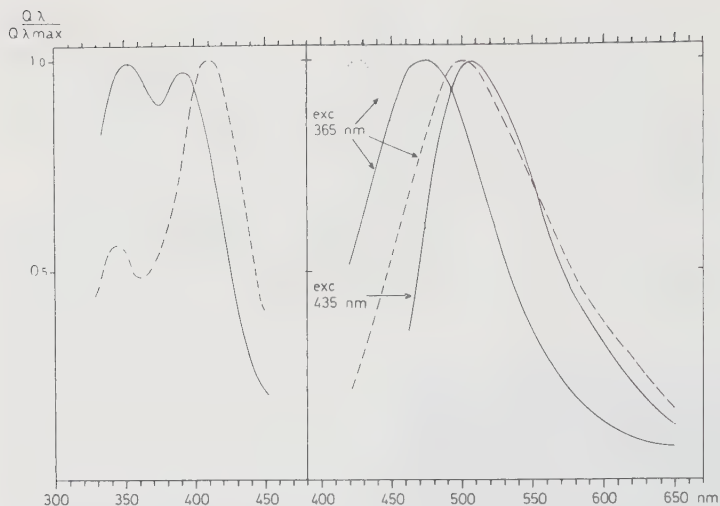


Fig. 2. Fluorescence excitation (left panel) and emission spectra (right hand panel) recorded in A11 cell bodies after injection of TB into the cervical spinal cord. (....) = cell body containing the TB label alone; (—) = cell body containing TB label and CA fluorescence; (---) = cell body in the ventral tegmental area containing CA fluorescence but no TB. The emission curve recorded at 435 nm excitation reveals the CA content of the TB-CA mixture.

large proportion of the diencephalic CA cell bodies which were retrogradely labelled from the spinal cord in the present study are dopamine-containing, a noradrenergic component in this projection cannot as yet be excluded.

Previous studies have shown that the A11 neurone system has rich intradiencephalic projections, i.e. to the medial and midline thalamus and several hypothalamic nuclei (probably including the periventricular, paraventricular and supraoptic nuclei and the mammillary bodies)^{2,14,15}. The present study shows that the same cell group has an important descending projection to the spinal cord in addition to these local intradiencephalic connections. Judging from the large proportion of the A11 neurones that were labelled by TB or PI injections into the spinal cord it seems highly probable that at least some of the neurones have both diencephalic and spinal cord projections, an arrangement that may be analogous to that described for the vasopressin-oxytocin neurones of the paraventricular nucleus^{7,18}. Interestingly, Lindvall et al.¹⁵ have observed that the axons of some A11 cells bifurcate in a T-shaped manner, giving rise to one ascending and one descending branch, thus suggesting that the intradiencephalic and the spinal cord projections could be established by collateral axonal branches of the same neurones. This interesting possibility will be the subject of further investigation.

In summary, combined monoamine histofluorescence and fluorescent retrograde tracing have shown that the vast majority of the A11 dopaminergic neurones, located

in the dorsal and posterior hypothalamus, zona incerta and caudal thalamus, project to the spinal cord. This hitherto unknown diencephalo-spinal dopaminergic projection is proposed to be a descending component of the dorsal periventricular CA system¹³, and a monoaminergic counterpart to the previously described hypothalamo-spinal peptidergic connections. The results, moreover, suggest that at least some of the A11 neurones give rise to both local diencephalic and descending spinal projections.

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ORIGIN AND TERMINATION OF THE DIENCEPHALO-SPINAL
DOPAMINE SYSTEM IN THE RAT

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Origin and Termination of the Diencephalo-Spinal Dopamine System in the Rat

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SKAGERBERG, G., A. BJÖRKLUND, O. LINDVALL AND R. H. SCHMIDT. *Origin and termination of the diencephalo-spinal dopamine system in the rat. BRAIN RES. BULL.* 9(1-6) 237-244, 1982.—Using a combination of neonatal 6-hydroxydopamine and adult 5,7-dihydroxytryptamine treatment we have been able to achieve a 94-99% depletion of noradrenaline in the spinal cord. In such animals the dopamine levels are only marginally affected in the dorsal horn (at all levels) and in the intermediate zone at thoraco-lumbar levels. This combined treatment thus offers new possibilities for selective studies of the spinal dopamine projection. In agreement with the biochemical data the fluorescence histochemistry shows that the spinal dopamine innervation is mainly confined to the dorsal horn, the intermediolateral cell column and associated parts of the intermediate and central gray. Injections of fluorescent retrograde tracer combined with monoamine fluorescence histochemistry reveal that the diencephalic A11 cell group is the principal, and perhaps exclusive, source of this innervation. The area of termination, as well as the organizational similarities with certain diencephalic peptide-containing projections to the spinal cord, suggest that the diencephalo-spinal dopamine system may be importantly involved in autonomic regulatory processes.

Diencephalo-spinal dopamine system Fluorescence histochemistry Spinal cord

RECENT biochemical studies have provided evidence that dopamine (DA) in the rat spinal cord is not only a precursor of noradrenaline (NA), as previously believed, but serves an independent transmitter role in a separate DA neuron system [8, 14]. This has been corroborated also with transmitter-specific retrograde tracing techniques, showing that DA-containing neurons in the diencephalon can be retrogradely labelled from injections into the spinal cord in rat and rabbit [6, 11].

From the biochemically measured DA and NA levels the spinal NA innervation can be assumed to be approximately 10-fold richer than the DA innervation. Due to technical limitations it so far has not been possible to distinguish this sparse DA innervation from the dense NA innervation.

In the present study we have made use of a combined hydroxydopamine (6-OHDA) and 5,7-dihydroxytryptamine (5,7-DHT) treatment in order to obtain a relatively selective fluorescence histochemical visualization of the spinal DA stores. In such pretreated animals we have been able to obtain evidence that the spinal DA innervation is mainly confined to the dorsal horn, the intermediolateral cell column and associated parts of the intermediate and central gray, and that the diencephalic A11 cell group is the principal, and perhaps exclusive, source of this innervation.

METHOD

Animals

Young adult male and female Sprague-Dawley rats (Anticimex AB, Stockholm, Sweden) were used.

Neurotoxin Treatment

The neurotoxin treated animals received two injections of 6-OHDA (Hässel AB, Göteborg, Sweden; 100 µg/g, SC each) at 1 and 2 days of age, followed by an intraventricular injection of 5,7-DHT (Regis; 150 µg) at 2-3 months of age. The doses were calculated as the free base. In the biochemical analyses littermates injected with saline were used as controls. The rats were killed 2-4 weeks after the last injection.

Biochemical Analyses

DA and NA were assayed radioenzymatically using a modification of the procedures described by DaPrada and Zürcher [9] and Saller and Zigmund [16]. This procedure, which is based on the use of catechol-O-methyl transferase and ³H-methyl-5-adenosyl-L-methionine, has been described in detail elsewhere [19].

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TABLE 1

DOPAMINE (DA) AND NORADRENALINE (NA) LEVELS (pg PER mg WET WEIGHT) IN THE SPINAL CORD OF UNTREATED LITTER-MATE CONTROL RATS, AND IN RATS TREATED WITH 6-OHDA ($2 \times 100 \mu\text{g/g SC}$) NEONATALLY AND WITH 5,7-DHT ($150 \mu\text{g}$ INTRAVENTRICULARLY) AT 3 MONTHS OF AGE

		Control			6-OHDA + 5,7-DHT Treatment		
		DA (pg/mg)	NA (pg/mg)	DA/NA ratio	DA (pg/mg)	NA (pg/mg)	DA/NA ratio
	Cervical cord dorsal (d)	35.8 $\pm$ 3.2	240 $\pm$ 9	0.15	26.6 $\pm$ 3.3‡	5 $\pm$ 2†	5.3
	ventral (v)	13.4 $\pm$ 2.4	197 $\pm$ 22	0.07	9.3 $\pm$ 2.9‡	1 $\pm$ 1†	9.3
	Thoracic cord dorsal (d)	24.6 $\pm$ 3.2	215 $\pm$ 20	0.11	30.1 $\pm$ 2.8‡	5 $\pm$ 2†	6.0
	intermediate (im)	39.0 $\pm$ 5.4	368 $\pm$ 10	0.11	34.4 $\pm$ 3.9‡	24 $\pm$ 2†	1.5
	ventral (v)	5.7 $\pm$ 0.8	96 $\pm$ 11	0.06	1.8 $\pm$ 0.9*	1 $\pm$ 1†	1.8
	Lumbar cord dorsal (d)	24.3 $\pm$ 1.2	331 $\pm$ 31	0.07	25.2 $\pm$ 0.9‡	5 $\pm$ 3†	5.0
	ventral (v)	8.6 $\pm$ 1.6	229 $\pm$ 13	0.04	1.7 $\pm$ 0.7*	2 $\pm$ 1†	0.85
	Lumbo-sacral cord dorsal (d)	28.2 $\pm$ 3.4	405 $\pm$ 37	0.07	24.3 $\pm$ 2.1‡	9 $\pm$ 4†	2.7
	ventral (v)	12.9 $\pm$ 1.0	321 $\pm$ 25	0.04	3.8 $\pm$ 1.1†	3 $\pm$ 2†	1.3

Means $\pm$ S.E.M. of 6 rats. Differences from control rats: * $p < 0.05$; † $p < 0.001$; ‡ $p > 0.05$. (Student's *t*-test). The schematic drawings show the dissection used.

Fluorescent Tracer Injections

True Blue (TB; trans-1,2-bis (5-amidino-2-benzofuranyl)-ethylen-2 HCl; kindly supplied by Dr. O. Dann, Erlangen, GFR) was injected in a 3% (w/v) suspension unilaterally into the cervical spinal cord in 3 rats pretreated with the combined neurotoxin injections. The injections were made over 15 min using a 1 μl Hamilton syringe equipped with a glass capillary tube glued to the needle. The injection volume was 0.3 μl . After 1–2 weeks survival the rats were processed for combined TB and catecholamine histofluorescence, according to Björklund and Skagerberg [5].

Histochemistry

Catecholamines were visualized using the ALFA method applied to freeze-dried and paraffin-embedded material [3]. In short, the animals were perfused with ice-cold Tyrodes buffer followed by c. 450 ml of the ALFA solution, perfused under high pressure. The entire spinal cord and brain stem was quickly dissected out, and selected pieces were rapidly frozen in a liquid propanepropylene mixture, cooled to between -80 and -90°C with liquid nitrogen. The pieces were subsequently freeze-dried at -35°C , reacted with formaldehyde vapours ($+80^\circ\text{C}$ for 1 hr, using paraformaldehyde equilibrated in 50% relative air humidity), and finally embedded in paraffin in vacuo and sectioned. For combined TB and catecholamine histofluorescence the pH of the ALFA

solution was adjusted to 5.0 through the addition of sodium acetate.

In the fluorescence microscope catecholamines were visualized at 435 nm illumination and TB (in the same section) at 365 nm illumination, following the principles outlined previously [5].

RESULTS

Biochemistry

The combined 6-OHDA and 5,7-DHT treatment caused a 94–99% reduction in NA in all parts of the spinal cord (Table 1). By contrast, the DA levels in the dorsal and intermediate cords were only marginally affected, exhibiting reductions of 25% or less. In the ventral cord, the DA levels were reduced by 70–80% in the thoracic, lumbar and lumbo-sacral segments, while the reduction was limited to about 30% at cervical levels (Table 1). As a result, the DA/NA ratio was increased from approximately 0.1 (in normal animals) to between 1.5 and 6.0 (in lesioned animals) in the dorsal and intermediate cord regions, indicating that most of the remaining DA in these regions is "NA-independent" and thus probably is localized in separate DA neurons. The residual NA levels were very low, except in the intermediate portion of the thoracic cord, where the NA content amounted to about 40% of the total catecholamine content.

The "NA-independent" DA was unevenly distributed in the cord (Table 1). The highest levels (25–35 pg/mg tissue)

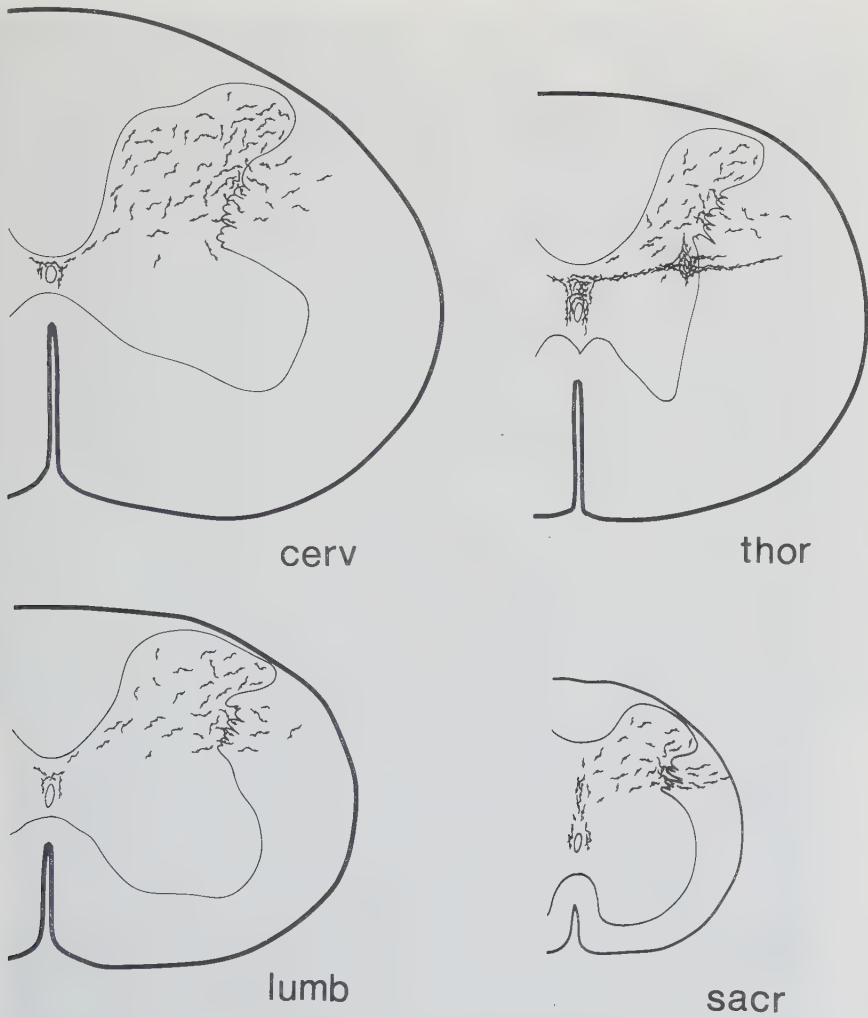


FIG. 1. Semischematic representation of the distribution of presumed dopamine fibres at four representative levels through the spinal cord of the rat.

ere recorded in the dorsal cord (at all levels) and in the thoracic intermediate cord. In the ventral cord, the DA levels were negligible (below 4 pg/mg) in the thoracic, lumbar and lumbosacral segments, while the concentration in the cervical ventral cord was intermediate (9 pg/mg).

Histochemistry

Consistent with the biochemical data, ALFA treated specimens revealed fluorescent catecholamine-containing fibres in the dorsal gray of the cervical, thoracic, lumbar and sacral cord, and in the intermediate gray of the thoracic cord.



FIG. 2. Photomicrograph showing presumed dopamine terminals in the dorsal horn of the cervical spinal cord from a rat subjected to the combined neurotoxin treatment.

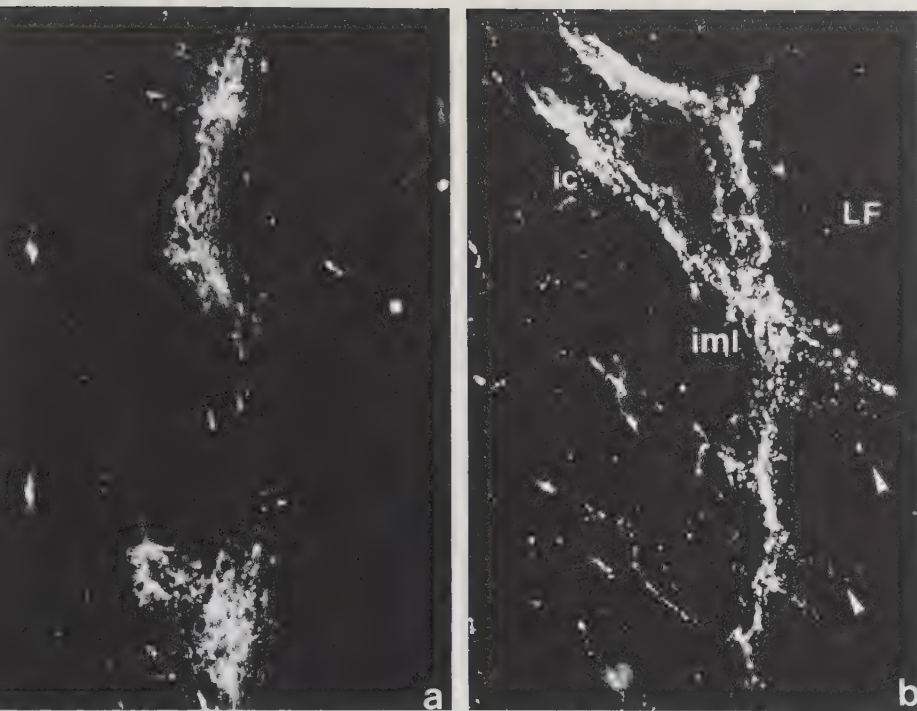


FIG. 3. Presumed DA-innervation of the intermediolateral cell column at mid-thoracic levels in a longitudinal section from a neurotoxin pretreated rat. a: Two patches of innervation along cell clusters in the intermediolateral column. ($\times 241$). b: Innervation of the intermediolateral cell column (iml), extending medially to the intercalated nucleus (ic). From the iml fibres are seen to extend laterally (arrowheads) into the lateral funiculus (LF). ($\times 241$).

the neurotoxin treated specimens. Their distribution is summarized semidiagrammatically in Fig. 1. In comparison with the normal specimens, processed in parallel, these presumed DA fibres represented only a minor portion of the total catecholamine innervation and they seemed to be fairly evenly distributed along the cord.

In the dorsal horn the presumed DA fibres were most abundant in the lateral parts of the superficial layers and in the adjoining reticular nucleus (i.e., the lateral part of lamina I, and in the adjoining dorsolateral funiculus. Some of these longitudinally running fibres appeared to give off collaterals towards the reticular nucleus and the dorsal horn.

The highest density of presumed DA fibres was found in

the intermediolateral cell column and the area surrounding the central canal at thoracic and upper lumbar levels (Fig. 3). Bands of fibres were also seen to interconnect the intermediolateral column, the intercalated nucleus and the central autonomic nucleus just dorsal to the central canal. This arrangement was particularly well demonstrated in longitudinal, horizontal sections. In such sections it could also be seen that the presumed DA innervation was not uniform along the intermediolateral column, but formed clusters or patches of varicose fibres, interconnected by thinner strands of more parallel running fibres. The fibre patches most likely correspond to the clustering of preganglionic sympathetic neurons along the intermediolateral column. Some coarse fluorescent fibres or fibre bundles were often seen to radiate out laterally from the intermediolateral cell column into the white matter, sometimes almost reaching the lateral surface of the cord. The distribution of the presumed DA

fibres in the intermediate zone thus has the same general arrangements as that described for the total catecholamine innervation in the cat by McLachlan and Oldfield [15].

The ventral horn was virtually free of fluorescent fibres in the neurotoxin treated specimens. Thus, only some single presumed DA fibres were found scattered in this area along the cord.

Fluorescent Tracer Injections

TB was injected unilaterally into the cervical spinal cord in rats pretreated with the combination of 6-OHDA and 5,7-DHT. As illustrated in Fig. 4, labeled DA-containing neurons were found ipsilaterally in the caudal diencephalon, in the so-called A11 cell group. These neurons are distributed along the dorsal and medial aspects of the mammillo-thalamic tract (Fig. 4a, b) and, more caudally, in the periventricular grey between the fasciculus retroflexus and the third ventricle (Fig. 4c). The labeling occurred also in many non-DA-containing neurons intermingled with the DA-containing ones. The overall pattern of TB labeling in the A11 area in the 6-OHDA/5,7-DHT pretreated animals was quite similar to that previously observed in normal rats [4].

A search for TB-labeled DA neurons in other cell groups—notably substantia nigra, the ventral tegmental area and the A13 cell group—gave a negative result.

DISCUSSION

The present combination of neonatal 6-OHDA and adult 5,7-DHT treatment is the only known treatment that can produce a >95% reduction of NA in areas like cerebral cortex and hippocampus (unpublished observations) and spinal cord, without substantial effects also on the DA levels. The neurotoxic effects of either the neonatal, subcutaneous 6-OHDA injections alone [18] or the intraventricular 5,7-DHT treatment alone are selective for the NA-containing neurons, but the reduction of NA obtained, e.g. in spinal cord, is maximally some 75–90%, which is insufficient for the present purposes. The results obtained with the combined 6-OHDA and 5,7-DHT treatment must, however, be interpreted with some caution:

First, despite substantial reductions, some NA remained in the cord. The residual concentrations were, however, negligible (1–9 pg/mg) in all parts except the intermediate thoracic cord. Here, the NA concentrations recorded (24 pg/mg) represented about 40% of the total catecholamine level in the area. Thus, even in the intermediate zone, where the highest density of fluorescent fibres was found in the neurotoxin pretreated animals, the majority of the remaining fluorescent structures can be expected to represent DA-containing ones.

Secondly, Hökfelt *et al.* [10] have immunohistochemically demonstrated the adrenaline-forming enzyme, phenylethanolamine-N-methyl transferase (PNMT) in the intermediolateral cell column in the thoracic cord, suggesting that adrenaline-producing neurons may also innervate this area. It is highly improbable, however, that any of the catecholamine fibres visualized in the neurotoxin pretreated animals are adrenaline-containing. Thus, the concentration of adrenaline in the cord is normally at least 5–10 times lower than that of DA [22,23]. Moreover, it is unlikely that any adrenaline-containing fibres can be visualized with the ALFA method [3].

Thirdly, the use of neurotoxin lesions in the present study makes it possible that the distribution of the remaining,

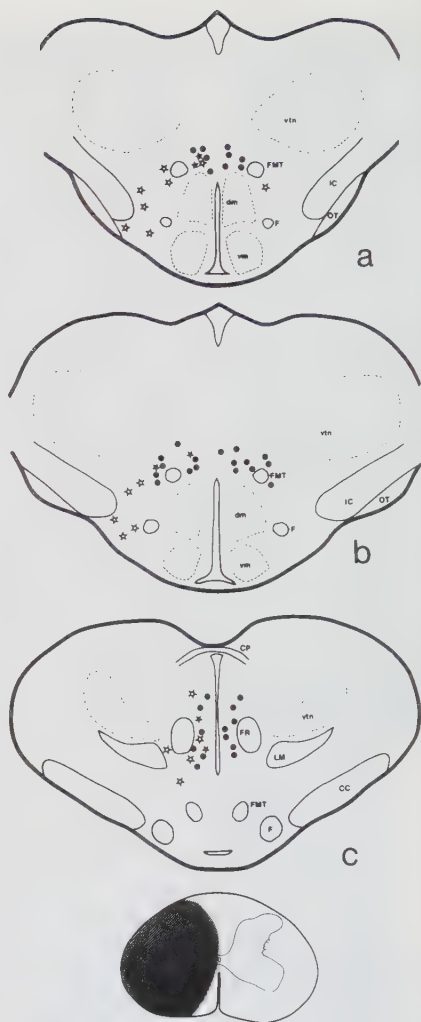


FIG. 4. Distribution of presumed DA- and True Blue containing cells in the diencephalon after unilateral cervical tracer injection. The shaded area in the cervical cord section represents the extension of the area of intense True Blue fluorescence at the injection site. Filled circles represent non-labeled CA-fluorescent cells. Open stars represent cells labeled with True Blue alone. Filled stars represent CA-fluorescent cells labeled with True Blue. Abbreviations: CC, crus cerebri; CP, commissura posterior; dm, dorsomedial nucleus; F, fornix; FMT, fasciculus mamillothalamicus; FR, fasciculus retroflexus; IC, internal capsule; OT, optic tract; vm, ventromedial nucleus; vtn, ventral thalamic nucleus.

THE DIENCEPHALO-SPINAL DA SYSTEM

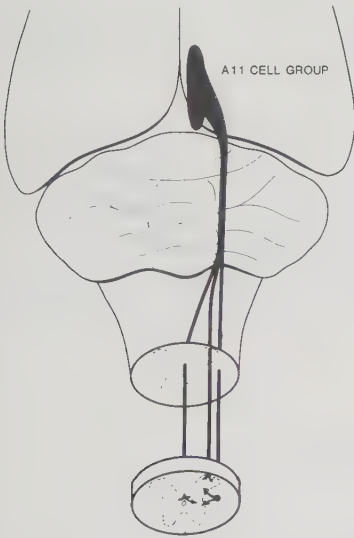


FIG. 5. Proposed arrangement of the diencephalo-spinal DA system as revealed in the neurotoxin-pretreated rats.

presumed DA-containing fibres may have undergone changes as a result of the previous denervation. At present it is not possible to control for this possible artifact. However, even if such changes had occurred in the specimens analysed, it is likely that the true distribution of the DA

innervation is at least not more extensive than that described presently.

With these reservations in mind it seems justified to conclude that the "NA-independent" catecholamine system revealed in the 6-OHDA and 5,7-DHT treated animals gives a good, even if not completely accurate, representation of the spinal DA system. As illustrated in this Fig. 5, which summarizes the present results, the descending spinal DA system seems to originate primarily, and possibly exclusively, in the diencephalic A11 cell group, forming a diencephalo-spinal DA pathway. This pathway appears to be totally uncrossed. The course of the descending fibres through the brain stem is not yet known. In the spinal cord the axons appear to descend partly within lamina I of the dorsal horn and the adjoining parts of the dorsolateral funiculus, and partly along the central canal. We propose that the former pathway gives rise to the DA terminals in the dorsal horn (particularly the lateral parts of the superficial layers and the reticular nucleus) at all levels of the cord, and that the latter pathway innervates the intermediolateral cell column and associated parts of the intermediate zone in the thoracic and upper lumbar segments. The arrangement of radiating fibres in the white matter lateral to the intermediolateral column may also suggest the possibility of DA fibres descending within the lateral funiculus. In fact, this proposed arrangement conforms well with the autoradiographic results of Saper *et al.* [17] after injections of tritiated amino acids into the posterior hypothalamus.

The proposed arrangement of the diencephalo-spinal DA system has an interesting similarity to that previously described for the diencephalo-spinal neurophysin I system [20,21] and the diencephalo-spinal somatostatin system [12]. These morphological similarities may reflect some functional interrelationships, and further support the involvement of the diencephalo-spinal DA system in autonomic regulatory processes.

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ORGANIZATION OF DIENCEPHALIC DOPAMINE NEURONS
PROJECTING TO THE SPINAL CORD IN THE RAT

G. Skagerberg and O. Lindvall

SUMMARY

Using the ALFA method for visualization of catecholamines in combination with injections of the fluorescent retrograde tracer True Blue (TB) we have studied those diencephalic dopamine (DA)-containing cell groups which have been proposed to give rise to the DA innervation of the spinal cord and investigated the organization of the diencephalo-spinal DA system in detail. The A13 cell group was found to contain 370, and the A11 cell group 140, DA-producing cells on each side, whereas only very few such cells were found in the paraventricular hypothalamic nucleus. Tracer injections into the spinal cord labelled only DA cells within the A11 group. The overall majority of labelled cells were found ipsilaterally but some cells were also found contralaterally indicating the existence of a minor crossed dopaminergic projection to the spinal cord. Large tracer injections which covered the hemichord at different levels generally resulted in very similar distributions and numbers of retrogradely labelled DA cells. The labelled DA-containing cells constituted 30-50% of the total number of labelled neurons in the ipsilateral A11 area and about 20-40% of the total number of DA containing cells in this area were labelled. Small injections that did not extend into the nucleus reticularis and the adjacent part of the lateral funiculus failed to label any diencephalic DA cells but usually labelled some non-DA cells in the A11 area.

It is concluded that the diencephalo-spinal DA neurons have long axons that extend over several segments and possibly traverse the entire length of the spinal cord, giving off collateral branches at various levels. From the anatomical data of the present study and previous pharmacological and electrophysiological findings it seems possible that diencephalo-spinal DA neurons could modulate both sympathetic activity and nociception.

Keywords:

A11 cell group - diencephalo-spinal system - dopamine - fluorescent retrograde tracing.

INTRODUCTION

The dopamine (DA)-producing neurons in the diencephalon are commonly divided into four subgroups^{5,12,16}: (1) the rostral periventricular cell group (A14) with neurons scattered in the periventricular hypothalamic region, from the level of the anterior commissure back to the rostral border of the median eminence; (2) the dorsal hypothalamic cell group (A13) comprising neurons clustered in the medial zona incerta, just ventro-medial to the mammillo-thalamic tract; (3) the tuberal cell group (A12) located in the arcuate nucleus and the adjacent part of the periventricular nucleus; and (4) the caudal cell group (A11) distributed in the posterior and dorsal hypothalamic areas and in the periventricular grey matter of the caudal thalamus. The best known projection from these neurons is the tuberohypophyseal DA system, which originates in the A12 cell group and innervates the median eminence and the neural lobe and pars intermedia of the pituitary^{4,14,15,30,45}. It seems likely that neurons in the other cell groups give rise to local diencephalic innervations, although these projections are less well established. The only extradiencephalic projection of the A11-A14 cell groups found so far is the diencephalo-spinal DA system^{7,8,9,11,23}, which, in the rat, has been found to innervate the dorsal horn and intermediolateral cell column of the spinal cord⁴³. The organization of this neuron system is presently known only to a very limited extent and there still is even some uncertainty about the exact localization of the cell bodies of origin. Thus, dopaminergic neurons projecting to the spinal cord have been described in the A11^{7,23,43} and A13⁸ cell groups as well as in the paraventricular hypothalamic nucleus⁴⁸.

The major objectives of the present study, which has been carried out with a retrograde tracing technique in combination with fluorescence histochemistry, were four-fold: First, to obtain some quantitative data on the number, size and distribution of DA-containing cells within the A11 and A13 groups and adjoining regions. Second, to describe the distribution of the cell bodies of origin for the dopaminergic diencephalo-spinal system. Third, to explore the possibility of a topographical organization within this system. Fourth, to obtain some quantitative data on the proportion of diencephalic DA cells innervating the spinal cord and to compare the number of dopaminergic and non-dopaminergic diencephalo-spinal neurons.

MATERIALS AND METHODS

Animals

About 25 female Sprague-Dawley rats (Anticimex AB, Stockholm, Sweden) were used.

Neurotoxin treatment

In order to remove the spinal noradrenergic system (see Skagerberg et al. 1982)⁴³ some animals were subjected to neurotoxin treatment. These rats received two injections of 6-hydroxy-DA (Hässlé AB, Göteborg, Sweden; 100 µg/g s.c.) at 1 and 2 days of age, followed by an intraventricular injection of 5,7-dihydroxytryptamine (5,7-DHT) (Regis; 150 µg) at 2-3 months of age. The doses were calculated as the free base.

Fluorescent tracer injections

Both neurotoxin treated and normal animals received injections of the fluorescent retrograde tracer True Blue (TB) when weighing about 200 g (i.e. about 3 weeks after the 5,7-DHT injection). The tracer injections were made at various levels between C₅ and S₁. Small volumes (0.1 µl TB, 3% in water freshly sonicated) were delivered through a glass capillary attached to a 1 µl Hamilton microsyringe. Larger injections (0.3-0.7 µl), aimed to fill the hemicord at the injected level were made directly through the original Hamilton cannula.

Histochemistry

After survival times ranging from 10 days to six weeks the animals were deeply anesthetized with chloral hydrate i.p. (0.5 g/kg) and perfused with 200 ml of ice-cold Tyrode's buffer and then with 350 ml ice-cold modified ALFA solution (2% paraformaldehyde + 5% Al₂(SO₄)₃ in phosphate buffer and pH adjusted to 4.7³⁵). Some normal rats that did not receive TB injections were also perfused in this manner. When the perfusion was completed the injected segment of the spinal cord as well as a tissue block comprising the di- and mesencephalon and overlying cortex were removed and rapidly frozen to the temperature of liquid nitrogen and subsequently freeze-dried for five days. The tissue pieces were then exposed to formaldehyde vapour and subsequently embedded in paraffin in vacuo, sectioned frontally or, in a few cases, sagittally at 15 µm. The sections were then deparaffinized and mounted in Entellan and examined in the fluorescence microscope.

Fluorescence microscopy.

Two different filter arrangements were used for providing suitable excitation wavelengths for TB or CAs. For excitation at 365 nm a Schott UG1 was used as primary filter sometimes in combination with a Zeiss 47 barrier filter or, alternatively, with no secondary filter. This filter setting optimally excites TB but also activates the CA-fluorophores. For excitation at 435 nm a Schott AL 435 served as primary filter and was always used in combination with Zeiss 47 as secondary filter. This filter setting allows for efficient detection of the CA fluorophores but does not, under normal circumstances, allow visualization of retrogradely transported TB^{6,22}.

The extent of two zones of the injection sites was delineated by shifting between the two filter settings⁴², and the segmental level of the injection was identified by comparing with Nissl-stained sections from recognized levels.

Cell counts were made by counting cell profiles in every third section and correcting this raw count by applying Abercrombie's formula¹.

Cell sizes were estimated in the microscope by using a measuring ocular and the positions of cells manually mapped onto outline drawings made from Nissl stained material obtained from other rats of the same size.

RESULTS

Localization and number of dopaminergic cell bodies in the A11 and A13 cellgroups and adjoining regions

In our experiments (see below), all labelled catecholamine (CA)-containing cell bodies were localized in the A11 cell group after tracers injections at different levels of the spinal cord. However, as pointed out in the Introduction, the spinal DA innervation has also been described to originate from the A13 cell group and the paraventricular hypothalamic nucleus. In the first part of the study we used the ALFA histofluorescence method to obtain some quantitative data on the distribution, number and size of CA cell bodies in these regions. Although the ALFA method does not differentiate between DA- and Noradrenaline (NA)-producing cells, since the overall majority of CA cells in the areas studied have been identified as DA-containing (see below), the cells exhibiting aldehyde-induced fluorescence will be described as dopaminergic.

In the paraventricular hypothalamic nucleus only very few dopaminergic cells were observed. The majority of these were found in the ventral part of the nucleus and were contiguous with the DA-containing cells located in the periventricular nucleus (A14 cell group). Other parts of the nucleus contained only single DA cells. It must be pointed out, though, that the dense catecholaminergic terminal innervation of the paraventricular nucleus makes it difficult to distinguish fluorescent cell bodies. Therefore, we might have underestimated the number of dopaminergic cells in this area. However, even when a major part of the dense fibre aggregation had been removed by the neurotoxin treatment (cf. Lindvall et al. 1984)³⁴ only few cells were found in the paraventricular nucleus.

The A13 cell group is located in the medial zona incerta ventromedial to the mammillo-thalamic tract (Fig. 1, level VII). The A13 cells are small and most often oval in shape with an average size of about 15 x 8 μm and with their long axis oriented medio-laterally. Most cells are densely aggregated but scattered neurons are distributed in rostral and lateral directions and there is no clear caudal boundary to the A11 cell group. We have estimated the antero-posterior extension of the compact part of the A13 cell group to be about 300 μm . An average of 369 ± 11 (means $\pm$ S.E.M, n=5) dopaminergic cells were counted in the A13 group on each side.

The A11 cell group is continuous with the cells located in the A13



Figure 1 Distribution of DA-containing cells as observed in seven coronal sections (approximately 300 μ m apart through the middle and caudal diencephalon of the rat. At the most rostral level (VII) the A13 group is schematically represented by a cluster of small dots. In the other sections the dots represent the total number of A11 cells as seen in one representative animal. The hatched line seen on the left side defines the A11 area (levels I - VI).

Abbreviations: CP, cerebral peduncle; DM, dorsomedial nucleus of hypothalamus; F, fornix; FMT, fasciculus mammillothalamicus; FR, fasciculus retroflexus; LHb, lateral habenula; MC, mammillary recess; OT, optic tract; PC, posterior commissure; SM, stria medullaris; SOX, supraoptic decussation; VM, ventromedial nucleus of hypothalamus.

group. Although there seems to be a gradual transition between the two cell groups, as revealed by cell morphology, we have chosen to define the border just caudal to the cell aggregation in the medial zona incerta. In Fig. 1 a representative example of the localization and number of dopaminergic cells in the A11 cell group is shown in six frontal planes through the diencephalon of a normal rat brain (Fig. 1, levels I-VI). The A11 cells are found scattered in dorsal and posterior hypothalamic areas extending dorsally along the periventricular grey of the caudal thalamus. In some brains occasional DA-containing cells were observed lateral and ventro-lateral to the mammillothalamic tract. The average number of A11 DA cells was found to be 136 ± 8 (means $\pm$ S.E.M., $n=11$) on each side. The majority of these cells is located rostrally, with the number gradually tapering off in the caudal half of the A11 group (Figs. 1 and 5). The predominant cell type in the A11 group is multipolar and about 25×15 – 20 μm in size, as observed in the coronal plane; in the sagittal plane the cells appeared slightly longer. Caudal to the A13 cell group, ventromedial and medial to the mammillothalamic tract we found a minor population of very weakly fluorescent, small, oval cell generally without discernable processes. Judged by their location and appearance these cells obviously do not belong to either the A11 or the A13 cell groups and were therefore not included in the cell counts.

Localization and number of retrogradely labelled, dopaminergic and non-dopaminergic cells after spinal tracer injections

In the second part of this study we used injections of the retrograde tracer TB at various spinal levels in combination with CA fluorescence histochemistry to locate diencephalic DA neurons projecting to the spinal cord. All injected brains were first analyzed with respect to the presence of retrogradely labelled cells in the different DA cell groups (A8–A14). After TB injections at all levels of the spinal cord practically all labelled DA cells were found in the A11 group (Fig. 2). A single labelled non-dopaminergic cell located among the A13 DA cells could be seen in some brains, and in one case we found one labelled periventricular DA neuron in the A14 group. No labelled DA cells were found in the A8, A9 and A10 cell groups after any of the spinal cord injections. In agreement with others^{18,40,38,19,46} we found a large number of labelled cells in the paraventricular hypothalamic nucleus and in the lateral hypothalamus

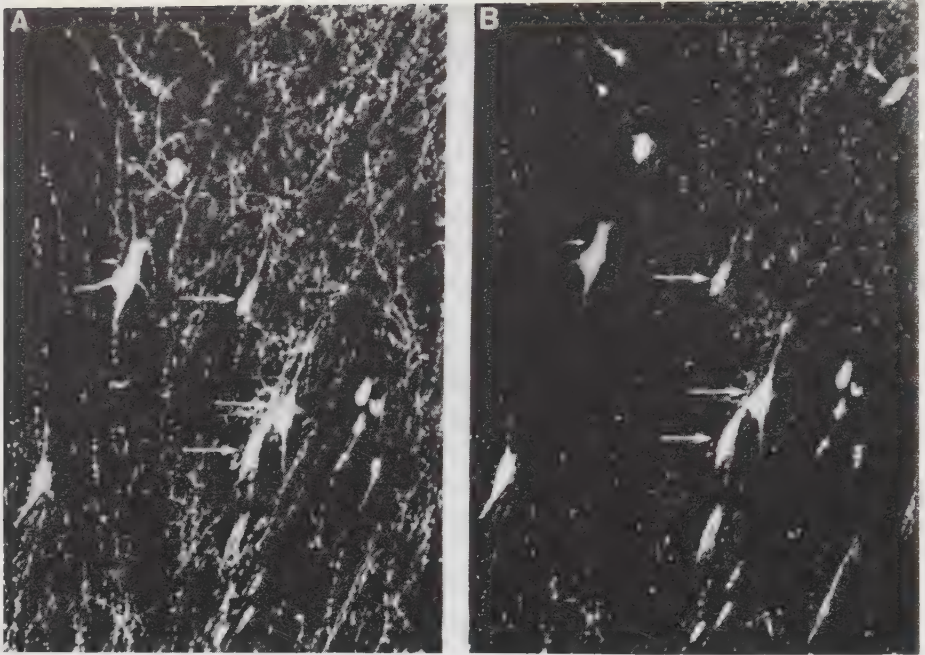


Figure 2 Example of retrograde labelling in the A11 area after an injection of TB in the spinal cord.

A. A cluster of A11 DA-containing cells, approximately at level III (see Fig. 1). Note the often multipolar appearance of these cells and the rich supply of CA axons in this area. 435 nm excitation.

B. The same area when excited at 365 nm, which reduces the CA fluorescence but optimally visualizes TB. Most of the DA cells also contain TB as evidenced by their increased fluorescence intensity, (some indicated by arrows). One DA cell shows less fluorescence (small arrow) and has thus not accumulated the tracer. Several cells or cell fragments that contain TB but not DA are also seen (arrow head). X290.

but none of these cells were DA-containing.

Since the labelled DA cells were confined to the A11 cell group we carried out a more detailed quantitative analysis of the labelled dopaminergic and non-dopaminergic cells in the area of this cell group ("A11 area"). The A11 area, as defined in the present study, is shown in Fig. 1. The findings in some representative brains after large injections of True Blue at different levels of the spinal cord are summarized in Figs. 3 and 4. These injections were all confined to one half of the spinal cord. The distribution of the labelled non-DA and DA cells is illustrated at 6 frontal levels through the diencephalon. After cervical, thoracic (not shown), lumbar and sacral injections labelled cells were distributed throughout the A11 area. Despite the fact that these injections were made at different levels of the spinal cord, the distributional pattern and number of labelled cells were very similar in the various experiments. The neurotoxin-treatment, which previously has been shown to remove selectively the NA innervation in the spinal cord⁴³, did not affect the number of labelled and non-labelled CA neurons in the A11 area. The labelled dopaminergic neurons were all of the multipolar A11 cell type. In contrast to the total population of A11 cells, most of which are found rostrally, the labelled DA cells were more evenly distributed along the antero-posterior extent of the A11 group (Figs. 3-5). They thus seemed to constitute a relatively greater part of the population of A11 cells at caudal levels. From the cell counts shown in Figs. 3 and 4 it can be estimated that the DA cells constitute 30-50% of the total number of labelled neurons in the ipsilateral A11 area. These data also show that 20-40% of the dopaminergic A11 neurons were labelled after injections at various spinal cord levels.

In addition, a minor population of retrogradely labelled DA and non-DA neurons was sometimes found in the A11 area contralateral to the injection site. The number of labelled dopaminergic cells on the contralateral side never exceeded 10% of that found ipsilaterally and was after most injections less than 5%. The non-DA projection from the A11 area could, on the other hand, be shown to be at least 50% crossed, provided that the tracer injections covered the most medial parts of the hemichord at S_1 (Fig. 4B).

In a previous histofluorescence study we have demonstrated that the DA innervation of the spinal cord is located in the dorsal horn, inter-

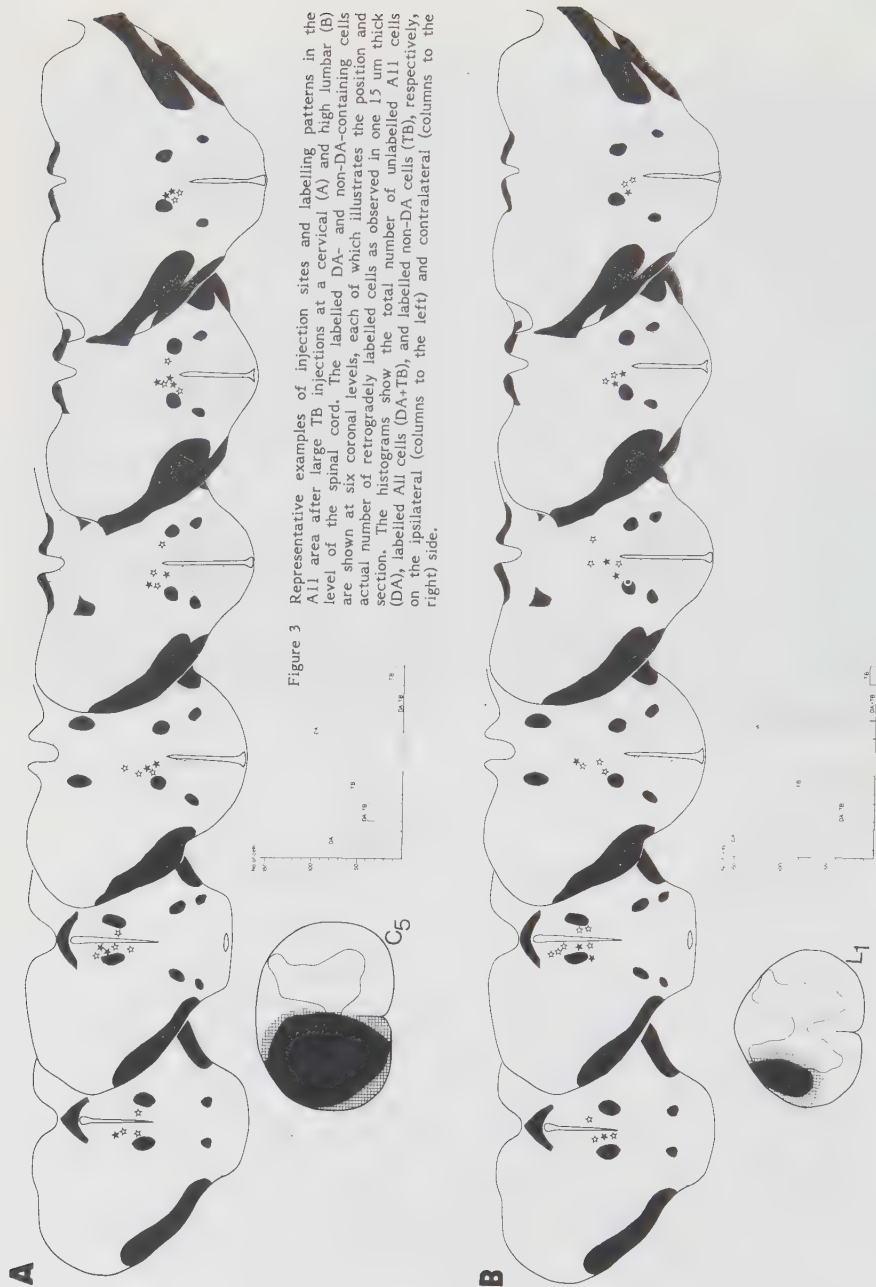


Figure 3

Representative examples of injection sites and labelling patterns in the A11 area after large TB injections at a cervical (A) and high lumbar (B) level of the spinal cord. The labelled DA- and non-DA-containing cells are shown at six coronal levels, each of which illustrates the position and actual number of retrogradely labelled cells as observed in one 15 μ m thick section. The histograms show the total number of unlabelled A11 cells (DA), labelled A11 cells (DA+TB), and labelled non-DA cells (TB), respectively, on the ipsilateral (columns to the left) and contralateral (columns to the right) side.

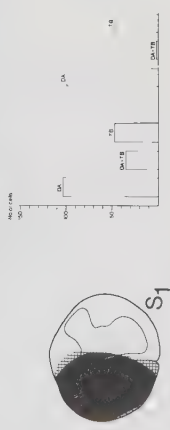
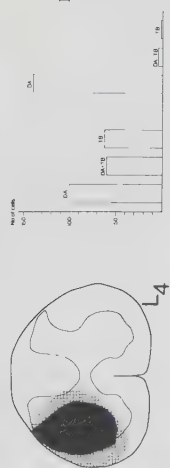


Figure 4 Representative examples of injection sites and labeling pattern in the A11 area after large TB injections at a low lumbar (A) and a sacral level (B), i.e. below the levels of the sympathetic preganglionic neurons. Symbols and histograms as in Fig. 2.

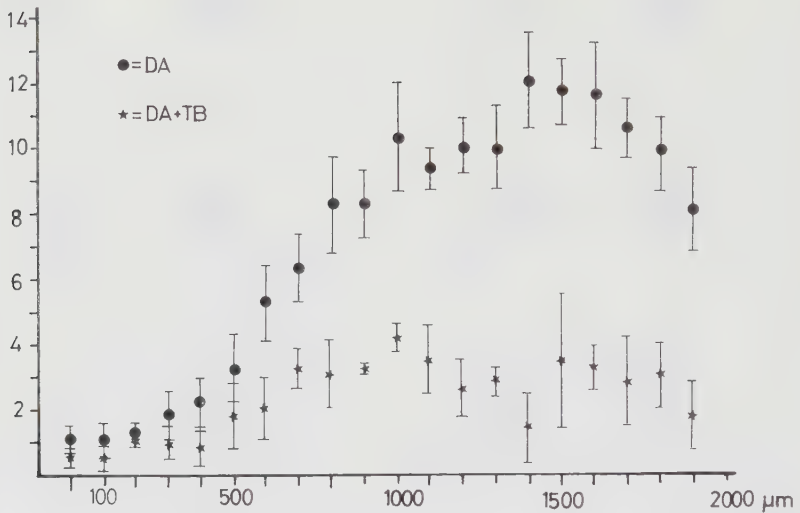
no. of cells/100 μm 

Figure 5 Rostrocaudal distribution of DA-containing cells in the A11 area (filled circles). Each filled circle represents the mean number of cells as seen per 100 μm (means $\pm$ S.E.M., $n=10$). Cells were counted starting immediately caudal to level I (Fig. 1), and proceeding rostrally to about half way between levels VI and VII. Similarly, the filled stars denote the average number of retrogradely labelled DA cells at various levels of the A11 group following large tracer injections into the spinal cord ($n=5$).

mediolateral cell column and along the central canal⁴³. We therefore made a series of smaller True Blue injections into different subregions in order to further explore the possibility of a topographical organization of the diencephalo-spinal DA projection (Fig. 6). However, neither small injections close to the central canal (Fig. 6D) nor in the intermediolateral cell columns (Fig. 6C) labelled any DA cells in the diencephalon. Single labelled non-dopaminergic neurons were found in the A11 area after the injections around the central canal. An injection into the ventral horn (Fig. 6E) labelled no A11 cells. While small TB injections which included the nucleus reticularis and the adjacent dorsal part of the lateral funiculus (Fig. 6A) gave rise to a distribution of labelled diencephalic DA cells similar to that seen after the larger hemicord injections (cf. above), tracer injections of similar size confined to the dorsal horn without extending into the nucleus reticularis or lateral funiculus did not result in any labelling of A11 cells.

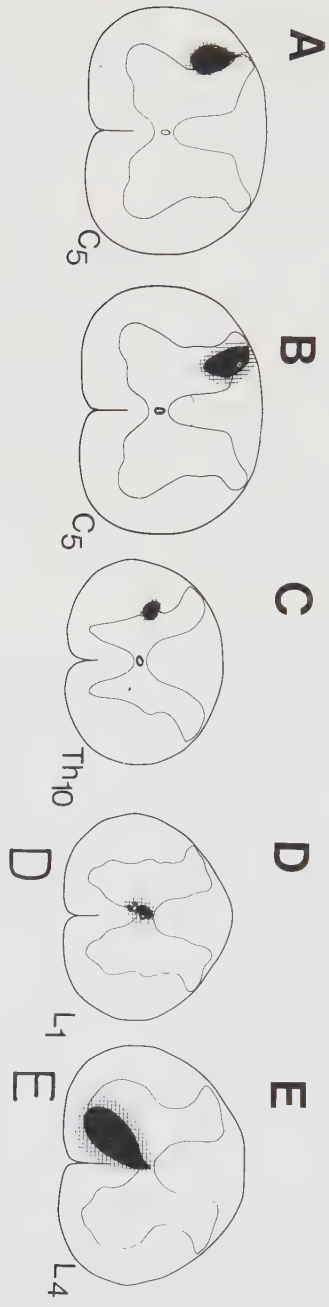


Figure 6 Schematic representations of some small TB injection sites. Only the injection at C₅ (A), which was located in the nucleus reticularis and the adjacent lateral funiculus, produced retrograde labelling in DA-containing A11 cells while the other labelled only a few non-DA cells in the A11 area.

DISCUSSION

The present study with the ALFA histofluorescence method has shown a distribution of CA cells in the A13 and A11 groups of the rat brain similar to that demonstrated previously with less sensitive fluorescence histochemical techniques^{5,25}. For the first time, quantitative data on the number of CA-containing neurons in these cell groups have been obtained. The A13 group was found to contain about 370 cells and the A11 group about 130 cells on each side. The majority of A11 neurons were distributed in the rostral half of the cell group. The total number of cells in the A11 and A13 groups (about 500) is similar to that estimated for the tuberal A12 cell group (about 500 on each side; as estimated by Björklund and Lindvall³, based on data from Lichtensteiger³⁰), which is the source of the tuberohypophyseal and tuberoinfundibular DA projections. Although the A11 and A13 groups thus should be considered as major diencephalic cell systems they are very small in comparison with the mesencephalic DA cell groups. These cell groups have been estimated to contain 15-20000 neurons on each side with about half in the A10 and half in the substantia nigra proper (A9; for refs. see Björklund and Lindvall 1984³). This means that the number of cells in, e.g., the A11 group only amounts to about 1-2% of that in the substantia nigra or A10.

There is a general agreement that all CA-containing cells in the A13 group are dopaminergic^{5,24}. Björklund and Nobin⁵ obtained microspectrofluorometric evidence for both DA- and NA-containing cells in the A11 group but these findings have later been challenged by immunohistochemical observations. While the distribution of tyrosine-hydroxylase-positive cells in the A11 area coincides closely with that of catecholaminergic cells as demonstrated with aldehyde histofluorescence^{5,24,36}, no DA- β -hydroxylase-containing cells (indicative of noradrenergic neurons) have been found in this area^{36,47}. It thus seems clear that the overall majority, if not all, of the A11 cells are dopaminergic. The present data show that the number of retrogradely labelled A11 cells is not affected by severe neurotoxin-induced depletion of spinal NA (about 94-99% reduction⁴³) and therefore support that at least the spinal projection neurons in the A11 group are DA-containing.

The ALFA method demonstrated only few CA-containing cells in the paraventricular hypothalamic nucleus, even if the major part of the dense NA innervation in this nucleus had been removed by neurotoxin treatment. This is in good agreement with previous fluorescence histo-

chemical observations^{5,25}, but differs markedly from observations with tyrosine hydroxylase immunohistochemistry. Swanson and co-workers⁴⁸ thus found more than 500 tyrosine-hydroxylase-stained cells in the paraventricular nucleus on each side. Similarly, Hökfelt et al.^{20,21} have described a fairly extensive tyrosine-hydroxylase immunoreactive cell system in the dorsal and ventral preoptic region, extending caudally into both the supraoptic nucleus and the ventrolateral hypothalamic area, which is not visible in ALFA-treated specimens. The reason for the discrepancy between the aldehyde fluorescence histochemical and immunohistochemical findings is unclear and can at present only be speculated upon. The cells might be dopaminergic but contain too low levels of DA to be visualized with the ALFA method. Some support for this explanation is given by the work of Lidbrink and co-workers³¹, who found a group of dopa-accumulating cells in the paraventricular nucleus. They concluded that these cells, which cannot be visualized in the untreated animal, could possibly be dopaminergic. Another possibility is that the paraventricular cells are not dopaminergic but that the tyrosine hydroxylase antibody might have stained an antigenically similar protein, rather than tyrosine hydroxylase in these cells. More work is obviously needed to clarify these issues.

In the present experiments True Blue injections performed at different levels of the spinal cord labelled only DA neurons belonging to the A11 cell group. Labelled DA cells confined to the A11 group have previously been reported after cervical^{7,43}, and lower thoracic and upper lumbar²³ tracer injections. No DA neurons of the A13 group were found to project to the spinal cord of the rat as has previously been described in the rabbit⁸. Most likely this discrepancy indicates species differences in the localization of the A13 and A11 cell groups, or possibly in the origins of the spinal DA innervation. Indeed, from the present work it seems reasonable to propose that the spinal projection is a characteristic feature of the A11 cell group, regardless of the exact localization of the cell bodies in different species. Although we found labelled neurons in the paraventricular hypothalamic nucleus none of these cells were DA-containing according to the ALFA method. However, Swanson and co-workers⁴⁸ have reported that some of the cells in this nucleus which are labelled after spinal injections are tyrosine hydroxylase positive. As has been discussed above, the interpretation of these cells as dopaminergic cannot entirely be based on immunohistochemical findings and therefore the existence of a paraventriculospinal DA pathway remains to be established.

It has been proposed that substantia nigra gives rise to a dopaminergic innervation of the spinal cord¹⁰. This hypothesis is based on the findings that 6-hydroxy-DA in the substantia nigra causes a reduction of the DA level in the thoracic spinal cord and that electrical stimulation of the substantia nigra leads to increased concentrations of DA metabolites in the same region. We found no labelled cells in the substantia nigra after any of the spinal tracer injections and therefore do not agree with the interpretation made by Commissiong and co-workers¹⁰. It seems more likely that the effects of their lesions and stimulations were due to interference with the function of dopaminergic axons originating more rostrally in the A11 region (cf. Hökfelt et al. 1979²³) or through indirect influences on spinal DA metabolism.

The diencephalo-spinal DA pathway only constitutes a minor portion of the hypothalamo-spinal projection system. The majority of labelled hypothalamic neurons after spinal tracer injections have been found in the paraventricular nucleus and lateral hypothalamic area^{18,19,38,40}. Hosoya¹⁹ reported that 8-16% (mean=12%) of all labelled hypothalamic neurons after injections into the spinal cord are located in the "dorsal and posterior hypothalamic areas" (which correspond to the "A11 area" in our nomenclature). From the present data and those of Hosoya (1980) it can be estimated that about 4-6% of the hypothalamo-spinal projection neurons are DA-containing. The overall majority of DA neurons projects ipsilaterally to the spinal cord but we also obtained some evidence for a minor, previously unknown, crossed dopaminergic projection. The number of labelled cells in the contralateral A11 group varied between 0 and 7% of that found ipsilaterally.

This study shows that the DA innervation at all levels of the spinal cord originates in multipolar neurons rather evenly distributed throughout the A11 cell group. Similar to what has previously been reported for all spinal projection neurons in the "dorsal and posterior hypothalamic areas"¹⁹, we found no topographical arrangement of the DA-containing ones in the rostrocaudal direction. About 20-40% of the A11 DA cells were labelled after large TB injections confined to one segment of the spinal cord regardless of the level of injection. Thus even large TB injections into the sacral cord labelled about 30% of the A11 DA cells. Very similar labelling was also obtained after small injections provided that they included the reticular nucleus and adjacent part of the lateral funiculus. In contrast, small injections that were confined to the grey matter did in all cases fail to retrogradely label A11 cells, even if the

injections were localized in areas known to contain DA terminals. The finding of similar numbers of retrogradely labeled A11 cells irrespective of the level of tracer injection indicates that probably all the spinal projecting A11 cells send their axons through most of the length of the cord. The failure of small injections confined to the grey matter (and thus not involving the DA-axons of passage) to label A11 cells is most easily explained by postulating that the long main axons of the A11 cells give off collateral terminal branches at most or all segmental levels. Small injections in the grey matter would then presumably only result in uptake of tracer from a very small fraction of the total terminal area of each neuron. This would result in the accumulation of very small and perhaps undetectable amounts of tracer in the parent cell body²⁹. While the DA neurons of the forebrain are generally considered to be topographically organized and to collateralize in a very restricted terminal area (see, e.g., Lindvall and Björklund 1983³²) the diencephalo-spinal DA cell thus seems to represent a new type of dopaminergic neuron with long, more collateralized axons distributed over wider terminal areas.

Knowledge about DA functions in the spinal cord is still limited and the physiological role of the diencephalospinal DA system can therefore merely be speculated upon. The dense dopaminergic projection to the intermediolateral cell column⁴³, as well as its origin in hypothalamic regions, probably indicates important functions for this system in the regulation of sympathetic outflow⁴⁴. Some support for this hypothesis is given by the previous findings that systemic administration of dopa or DA receptor antagonists to spinal animals influences sympathetic nervous activity^{2,37}. More direct evidence for a modulatory role of intraspinal dopaminergic mechanisms on sympathetic outflow were recently reported by Simon and Schramm⁴¹. Using a spinal superfusion technique they found that DA increased spontaneous renal sympathetic nerve activity via specific stimulation of spinal dopaminergic receptors. Furthermore, they observed that depletion of spinal DA, but not NA or adrenaline, reduced renal nerve excitation elicited by electrical stimulation in laminae 4 to 7 of the spinal grey, and that subsequent superfusion with DA, but not with NA, potentiated this effect. Interestingly, electrical stimulation in the dorsal and posterior hypothalamus, i.e. areas which contain the cell bodies of origin for the spinal DA innervation, has been reported to increase sympathetic nervous activity¹⁷. From these observations it seems possible that at least part of the diencephalospinal DA neurons form a sympatho-

excitatory system.

The concentration of DA terminals in the dorsal horn suggests a modulatory role for the diencephalospinal DA system in the processing of sensory information, such as pain. Many experimental data indicate an involvement of central dopaminergic mechanisms in nociception (for references, see Lindvall et al. 1983³²). Available evidence from studies with various nociceptive tests suggest that spinal dopaminergic mechanisms have an inhibitory effect on noxious input to the spinal cord. Intrathecal administration of apomorphine into the lumbar subarachnoid space but not into the lateral cerebral ventricle produces a dose-dependent antinociceptive effect in intact animals as measured by the thermally evoked hot plate response and the acetic acid elicited writhing response²⁸. Furthermore, intrathecally administered apomorphine and DA have been reported to increase the tail flick latency in spinal rats^{26,27}. The effects of apomorphine and DA observed in these studies were counteracted by the previous administration of DA receptor antagonists^{26,28}.

It is well established that electrical stimulation in midline periventricular sites of, e.g., thalamus and hypothalamus can produce analgesia in a variety of species including man¹³. In fact, stimulation-produced analgesia has been elicited from part of the area of the A11 cell group³⁹. Together with the pharmacological data summarized above this suggests that diencephalospinal DA neurons might constitute an endogenous pain inhibitory system.

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**SELECTIVE HISTOCHEMICAL DEMONSTRATION OF DOPAMINE
TERMINAL SYSTEMS IN RAT DL- AND TELENCEPHALON:
NEW EVIDENCE FOR A DOPAMINERGIC INNERVATION
OF HYPOTHALAMIC NEUROSECRETORY NUCLEI**

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Selective Histochemical Demonstration of Dopamine Terminal Systems in Rat Di- and Telencephalon: New Evidence for Dopaminergic Innervation of Hypothalamic Neurosecretory Nuclei

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The distribution of dopamine (DA)-containing fibers in the virtual absence of noradrenaline (NA)-containing ones has been mapped by aldehyde fluorescence histochemistry in rats subjected to a combined neurotoxin treatment (intracerebral 6-hydroxydopamine injections plus systemic injections of the selective NA neurotoxin DSP-4). This pretreatment left di- and telencephalic DA levels largely unaffected, but reduced the NA levels by at least 86–96%. The resulting DA:NA ratios suggested that the catecholamine-containing structures, demonstrable by fluorescence histochemistry in the di- and telencephalic regions, were predominantly the DA-containing ones.

While the distribution of DA terminal systems in the neo- and allocortical regions conformed well to previous results, the combined neurotoxin treatment revealed new features of the distribution of DA fibers in the diencephalon. In addition to the previously described innervations of the tubero-hypophyseal system, the incerto-hypothalamic system, and the mesohabenular pathway, previously unknown innervations were revealed in the supraoptic, paraventricular and dorsomedial nuclei of the hypothalamus, and in the paraventricular nucleus of the thalamus. Apart from some scattered fibers in the periventricular and lateral hypothalamic areas and medial zona incerta, other diencephalic nuclei seemed to be devoid of any significant DA terminal networks. The dopaminergic nature of these innervations is supported by DA uptake experiments (evaluated by fluorescence histochemistry) as well as by independent biochemical and immunohistochemical evidence.

It is suggested that the DA innervations of the hypothalamic neurosecretory nuclei originate in cell bodies of the diencephalic A11–A14 cell groups and that such intradiencephalic DA projections participate in the regulation of oxytocin and vasopressin release from the pituitary.

INTRODUCTION

Dopamine (DA) in the di- and telencephalon was originally associated with 3 neuronal systems having rather restricted projection areas: the tubero-hypophyseal, nigrostriatal and mesolimbic systems³⁹. During the last decade, DA innervations have been found to be more widespread (see e.g. ref. 21), but although our knowledge of the anatomy of forebrain DA systems has thus expanded considerably, there are still some areas where the organization of the DA input is less well known. This is true in particular for several regions of the diencephalon which are outside the projection field of the well-known tubero-hypophyseal DA system, but which nevertheless have been shown by biochemical assays to contain higher

DA levels than would be expected if DA acted solely as a precursor of NA^{30,40}. Of special interest in this context are the supraoptic and paraventricular nuclei of the hypothalamus. There is considerable pharmacological and physiological evidence that DA is acting on neurons in these nuclei to regulate the release of both vasopressin and oxytocin from the posterior pituitary^{27,28}. A direct dopaminergic innervation of the hypothalamic neurosecretory nuclei has, however, so far not been demonstrated.

The difficulties in distinguishing the cellular localization of the presumed dopaminergic innervations in the diencephalon are mainly due to the presence of noradrenaline (NA) terminal systems in the same regions, which, on the basis of biochemical determinations of catecholamine (CA) levels, can be esti-

mated to be 5–10 times denser than the DA-containing ones. Since the aldehyde histofluorescence methods do not directly differentiate between DA- and NA-containing structures, the microscopic identification of DA neurons in such areas requires the removal of major parts of the NA input. However, the lesion techniques used so far have not caused sufficiently complete depletion of NA without simultaneous damage to the DA systems. This paper describes a new combined lesion procedure using bilateral 6-hydroxydopamine (6-OHDA) injections into the ascending NA bundles in the mesencephalon, followed by two systemic injections of the NA-selective neurotoxin DSP-4¹⁷. With this experimental approach, which leaves the DA systems unaffected, NA levels in hypothalamic and thalamic regions are decreased by 86–96%, and in the telencephalon, the amount of residual NA is negligible. Using this procedure we now report evidence for a dopaminergic innervation of the hypothalamic supraoptic, paraventricular and dorsomedial nuclei, as well as of the paraventricular thalamic nucleus and the lateral habenula. Furthermore, we demonstrate the usefulness of this procedure for selective and detailed studies on telencephalic DA terminal patterns, e.g. in the amygdaloid–piriform region.

MATERIALS AND METHODS

Neurotoxin treatment

Young adult female Sprague–Dawley rats (180–200 g body weight; Anticimex AB, Stockholm, Sweden) were used. Under general barbiturate anaesthesia (Brietal, Lilly, 40 mg/kg), they were first given 6-OHDA bilaterally into the central tegmental tract at the level of the caudal mesencephalon (8 µg in 4 µl, two injections on each side; for details, see ref. 25). With the tooth-bar set at 0, the coordinates were 0.4 anterior and 1.3 lateral. For the vertical coordinates, the needle was lowered through the brain to the base of the skull and was then withdrawn dorsally 2.5 mm, where the first injection was given, and then further elevated 1.0 mm, where the second injection was given. One week later the animals were injected intraperitoneally with DSP-4 (N-2-chloroethyl-N-ethyl-2-bromobenzylamine, AB Astra, Södertälje, Sweden, 50 mg/kg in physiological saline). The DSP-4 injection was repeated 5 days later. The

lesioned rats were killed 2 weeks after the last injection and then subjected to either biochemical or histochemical analysis, and using this compared to normal untreated animals.

Biochemical analysis

DA and NA were assayed radioenzymatically using a modification of the methods described by DaPrada and Zürcher⁹ and Saller and Zigmond³³. The procedure used in the present experiments, which is based on the use of catechol-O-methyl transferase and [³H]methyl-5-adenosyl-L-methionine, has



Fig. 1. Localization of the dissected tissue pieces taken for determination of DA and NA. APL, amygdaloid–piriform lobe; ERC, entorhinal cortex; H, hypothalamus; Ha, anterior hypothalamus; Hp, posterior hypothalamus; HPC, hippocampus; me, median eminence; TH, thalamus; THl, lateral thalamus; THm, medial thalamus.

been described in detail elsewhere³⁴. The regional dissection of the brain was carried out as schematically illustrated in Fig. 1.

Histochemical analysis

CAs were visualized using the ALFA method applied to freeze-dried and paraffin-embedded material²⁶. In short, the animals were perfused with ice-cold Tyrode's buffer followed by about 450 ml of the ALFA solution, perfused under high pressure (2 bar/cm²). The forebrain was quickly dissected out and divided into 2–4 smaller pieces to minimize cracking. The pieces were rapidly frozen in a liquid propane–propylene mixture, cooled to between –80 and –90 °C with liquid nitrogen. They were subsequently freeze-dried at –35 °C, reacted with formaldehyde vapor (at +80 °C for 1 h, using paraformaldehyde equilibrated in 50% relative air humidity), and finally embedded in paraffin in vacuo and sectioned.

Dopamine uptake experiments

Sections from fresh brain tissue of normal rats were cut with a Vibratome instrument and then incubated according to the method described previously²⁰ in 5×10^{-6} M DA for 20 min, with or without desipramine (10^{-5} M; Ciba–Geigy). The incubations were performed on sections from animals treated with reserpine (Serpasil, Ciba–Geigy, 5 mg/kg, 12–18 h before sacrifice) and nialamide (Pfizer; 300

mg/kg, 1–3 h before sacrifice). The sections were then processed according to the glyoxylic acid method²⁰ and examined by fluorescence microscopy.

RESULTS

The combined neurotoxin treatment (intracerebral injection of 6-OHDA in the caudal mesencephalon followed by two consecutive systemic injections of DSP-4) caused extensive reduction of forebrain NA. Table I shows CA levels in the 8 different regions taken for biochemical analysis at 2 weeks after the last DSP-4 injection. The NA depletion ranged from 86–87% in hypothalamus to 90–96% in thalamus and 96–99% in cortical regions. DA levels were, on the other hand, largely unchanged. Consequently, there was a general and pronounced increase in the DA:NA ratio (to between 1.6 and 10.7) after the combined neurotoxin treatment, indicating that the majority of CA fibers left in the regions analyzed were DA-containing. The remaining fluorescent CA fibers were subsequently mapped. In the following sections, the biochemical data and fluorescence microscopic findings will be described for each region.

General overview of telencephalic regions

Amygdaloid–piriform lobe. In this region, NA was severely depleted (96% decrease) with only minor changes in the DA level. The DA:NA ratio was 9.1, and so the remaining fibers should be almost exclu-

TABLE I

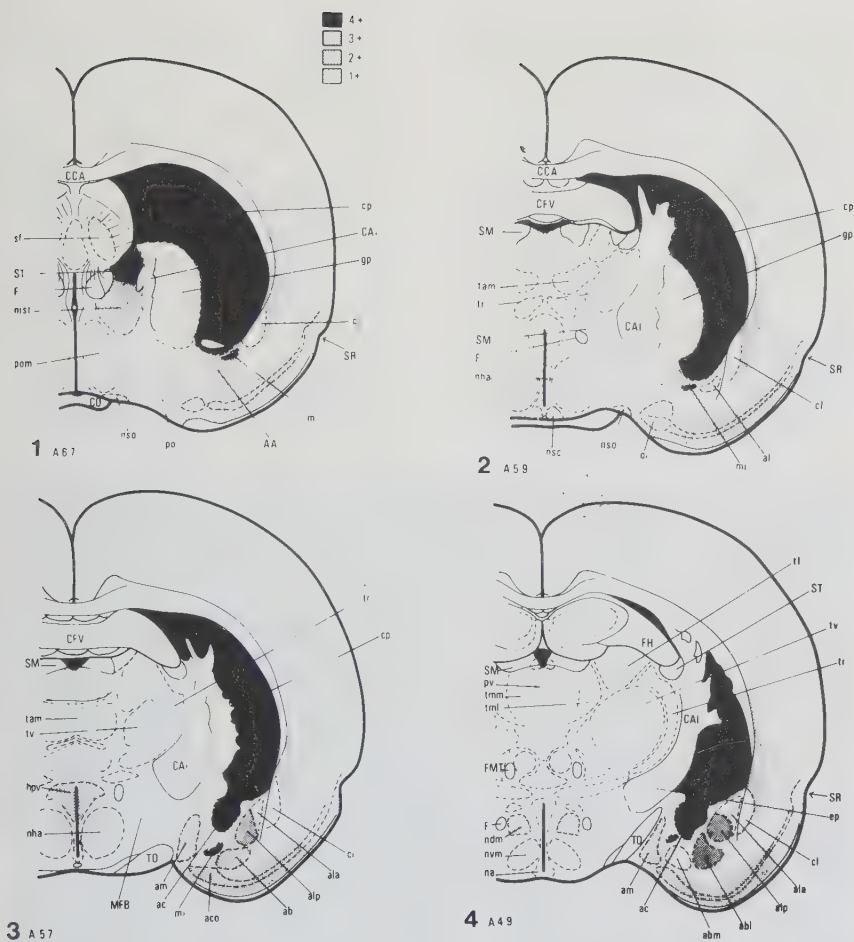
NA and DA concentrations and DA:NA ratios in 8 brain regions in normal rats and in rats subjected to the combined 6-OHDA and DSP-4 treatment

Means $\pm$ S.E.M. of 6–12 determinations

Region	Normal		DA:NA ratio	6-OHDA+DSP-treated ^a		DA:NA ratio
	DA (ng/mg tissue)	NA (ng/mg tissue)		DA (ng/mg tissue)	NA (ng/mg tissue)	
Hippocampus	0.022 $\pm$ 0.005	0.205 $\pm$ 0.007	0.11	0.011 $\pm$ 0.003	0.002 $\pm$ 0.001	5.50
Entorhinal cortex	0.035 $\pm$ 0.003	0.255 $\pm$ 0.010	0.14	0.045 $\pm$ 0.005	0.005 $\pm$ 0.002	9.00
Amygdaloid–piriform lobe	0.170 $\pm$ 0.046	0.334 $\pm$ 0.020	0.51	0.137 $\pm$ 0.021	0.015 $\pm$ 0.005	9.13
Anterior hypothalamus	0.254 $\pm$ 0.038	1.102 $\pm$ 0.086	0.23	0.261 $\pm$ 0.068	0.159 $\pm$ 0.015	1.64
Posterior hypothalamus	0.283 $\pm$ 0.039	0.593 $\pm$ 0.048	0.48	0.296 $\pm$ 0.024	0.080 $\pm$ 0.010	3.70
Median eminence ^b	7.173 $\pm$ 2.323	5.716 $\pm$ 0.938	1.26	11.805 $\pm$ 3.321	1.105 $\pm$ 0.271	10.68
Medial thalamus	0.052 $\pm$ 0.004	0.286 $\pm$ 0.009	0.18	0.050 $\pm$ 0.005	0.029 $\pm$ 0.008	1.72
Lateral thalamus	0.088 $\pm$ 0.021	0.252 $\pm$ 0.009	0.35	0.045 $\pm$ 0.014	0.011 $\pm$ 0.004	4.09

^a The rats were first given 6-OHDA locally into the central tegmental tract ($2 \times 8 \mu\text{g}$), followed 1 week later by two i.p. injections of DSP-4 (50 mg/kg each, 5 days apart). The rats were killed 2 weeks after the last injection.

^b The values for median eminence are expressed as ng/mg protein



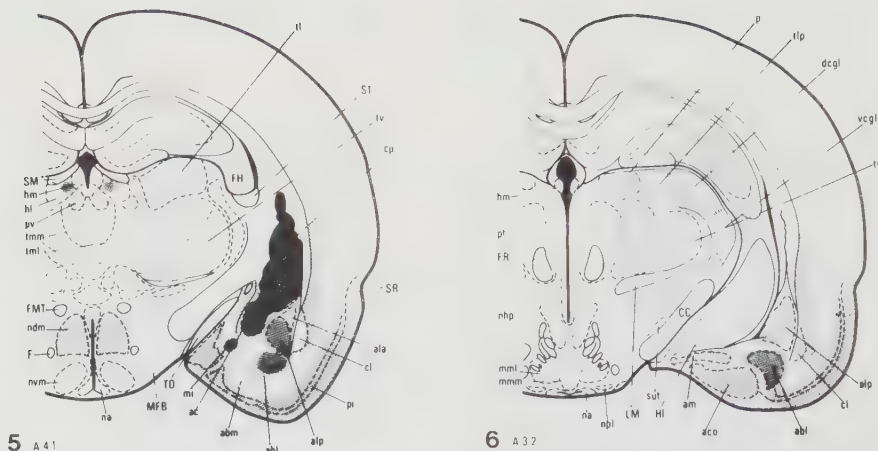


Fig. 2. Schematic representation in 6 frontal planes through the rat forebrain of the distribution and density of DA terminals as revealed by the present combined lesion procedure. The presumed DA innervations in the medial zona incerta and in the lateral hypothalamus-MFB area are not illustrated. The terminals of the incertohypothalamic and tuberohypophyseal systems (see e.g. refs. 5 and 6) have also been omitted. The terminal densities range from very dense (4+), dense (3+), medium dense (2+) to low density (1+). **Abbreviations:** AA, area amygdaloidea anterior; ab, nucleus amygdaloideus basalis; abl, nucleus amygdaloideus basalis, pars lateralis; abm, nucleus amygdaloideus basalis, pars medialis; ac, nucleus amygdaloideus centralis; aco, nucleus amygdaloideus corticalis; al, nucleus amygdaloideus lateralis; ala, nucleus amygdaloideus lateralis, pars anterior; alp, nucleus amygdaloideus lateralis, pars posterior; am, nucleus amygdaloideus medialis; CAI, capsula interna; CC, crus cerebri; CCA, corpus callosum; CFV, commissura fornicis ventralis; cl, claustrum; CO, chiasma opticum; cp, nucleus caudatus-putamen; dcgl, nucleus dorsalis corporis geniculati lateralis; ep, nucleus entopenduncularis; F, fornix; FH, fimbria hippocampi; FMT, fasciculus mamillothalamicus; FR, fasciculus retroflexus; gp, globus pallidus; HI, hippocampus; hl, nucleus habenulae lateralis; hm, nucleus habenulae medialis; hvp, nucleus paraventricularis hypothalami; LM, lemniscus medialis; MFB, medial forebrain bundle; mi, massae intercalatae; mml, nucleus mamillaris medialis, pars lateralis; mmm, nucleus mamillaris medialis, pars medialis; na, nucleus arcuatus; ndm, nucleus dorsomedialis; nha, nucleus hypothalamicus anterior; nhp, nucleus hypothalamicus posterior; nist, nucleus interstitialis striae terminalis; npl, nucleus prelateralis mamillaris; nsc, nucleus suprachiasmaticus; nso, nucleus supraopticus; nvm, nucleus ventromedialis; ol, nucleus tractus olfactorii lateralis; p, nucleus pretectalis; pf, nucleus parafascicularis; pi, cortex piriformis; pol, nucleus preopticus lateralis; pom, nucleus preopticus medialis; pv, nucleus paraventricularis thalami; sf, nucleus fimbrialis septi; SM, stria medullaris thalami; SR, sulcus rhinalis; ST, stria terminalis; sut, nucleus subthalamicus; tam, nucleus anterior medialis thalami; tl, nucleus lateralis thalami; tlp, nucleus lateralis posterior thalami; tml, nucleus medialis thalami pars lateralis; tmm, nucleus medialis thalami pars medialis; TO, tractus opticus; tr, nucleus reticularis thalami; tv, nucleus ventralis thalami; vcgl, nucleus ventralis corporis geniculati lateralis.

with the highest density medially (Fig. 3B). Moderately dense terminal arrangements were seen around cells in layer II. Scattered axons entered layer I, and deeper parts of the piriform cortex were innervated by a rather loose plexus of fibers.

Entorhinal cortex. The noradrenergic input was almost completely removed by the lesions (98% decrease in NA), and from the DA:NA ratio it can be concluded that practically all remaining fluorescent structures in the entorhinal cortex were dopaminergic. Consistent with previous descriptions^{13,23}, the majority of axons were aggregated in islands in the second and third layers of the most anterior portion

of the entorhinal cortex (Fig. 3A). In addition, a sparse but significant, more diffusely organized plexus of delicate fibers was distributed over more posterior parts of the entorhinal cortex.

Hippocampus. In the lesioned animals hippocampal NA was reduced by 99%, whereas DA was reduced by 50%, and the DA:NA ratio increased to 5.5. The DA level was very low in the hippocampus after the neurotoxin treatment (0.011 ng/mg) and in the dorsal hippocampus only some very few, scattered CA fibers were observed fluorescence microscopically (Fig. 4A). The density of fluorescent axons was somewhat higher in the ventral hippocampal

formation but even here, the remaining fiber plexus, which had a diffuse distribution, was very sparse.

Neocortex and perirhinal cortex. No measurements of CA levels were performed in these regions. However, it has been shown in other experiments (unpublished observations) that the 6-OHDA injection in the mesencephalon causes an almost complete removal of the neocortical NA innervation. Furthermore, DSP-4 in itself gives a more than 90% decrease in NA in the neocortex without affecting DA levels¹⁷. After the combined neurotoxin treatment, remaining neocortical CA fibers were localized in the frontal lobe and in the perirhinal cortex, whereas other neocortical areas contained no or only very few scattered axons.

In the frontal lobe the terminal plexa corresponded closely to what has previously been described as the anteromedial, suprarhinal and supragenual DA systems, respectively. A system of deli-

cate terminal fibers was observed mainly in the basal cortical layers, coinciding partly with the perirhinal DA system²². However, it was observed in this study that the perirhinal terminal system extended more dorsally than previously described, reaching about 2 mm above the rhinal sulcus (see Fig. 2). Rostrally, it merged with the suprarhinal system and caudally it could be followed into an area overlying the posterior (vertical) part of the hippocampus, i.e. a region that may be equivalent to the temporal association cortex⁴². The highest fiber density was found in the anterior parts of the system, and here, ventrally localized fibers were aggregated around the dorsal and lateral margins of the claustrum (levels 1–6 in Fig. 2). Going caudally (i.e. posterior to level 6), the density of terminals decreased considerably and in the temporal association cortex only a very sparse fiber plexus was observed in the basal cortical layers.



Fig. 3 Remaining CA-containing fibers in frontal sections through the entorhinal cortex (A) and medial piriform cortex (B) after the combined 6-OHDA-DSP-4 lesioning procedure. The DA:NA ratios indicate that the innervations shown are almost exclusively dopaminergic ($\times 150$).

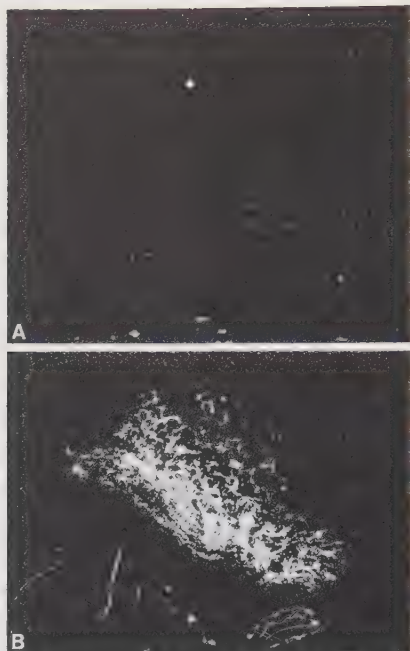


Fig. 4. A. Photomicrograph showing a typical example of the effects of the 6-OHDA-DSP-4 lesioning procedure on the CA innervation in the dorsal hippocampus (CA3 region). Only occasional scattered axons can be observed (frontal section; $\times 150$). B. Remaining CA-containing fibers in a frontal section through the supraoptic nucleus after the neurotoxin treatment. The DA:NA ratio suggests that a significant part of the terminals are DA-containing. Note that the terminals are concentrated in the ventral portion of the nucleus and that many fibers form basket-like arrangements around non-fluorescent nerve cells ($\times 140$).

Diencephalon

Hypothalamus. In the normal, untreated control animals, considerable amounts of DA were found biochemically in the hypothalamus, also outside the median eminence. The neurotoxin treatment left the DA levels unaffected but caused a fall in hypothalamic NA of 86–87%. This was observed in the fluorescence microscope as an extensive removal of CA-containing fibers. The denervation was, however, not uniform: the remaining axons were largely concentrated to some hypothalamic nuclei, whereas the

CA input to others had been almost completely removed.

For biochemical CA determinations, the hypothalamus was divided into two parts with a transverse cut through the rostral portion of the dorsomedial nucleus. Both in the normal and lesioned animals the DA:NA ratio was higher in the posterior (0.48 and 3.7, respectively) than in the anterior (0.23 and 1.64, respectively) part. The residual DA concentration was similar in the two parts (0.261 and 0.296 ng/mg), indicating that the presumptive DA innervation is fairly evenly distributed in the anterior and posterior hypothalamus.

Dense aggregations of fibers belonging to the incertohypothalamic DA fiber system (not included in the diagrams in Fig. 2) were found in the *medial preoptic region*, the *anterior hypothalamic nucleus*, *zona incerta* and in the dorsal part of the *dorsomedial nucleus*. In addition, the preoptic region as well as the ventral part of the *interstitial nucleus of the stria terminalis* and the region ventral to the anterior commissure contained a low density of coarser, presumably NA-containing terminals.

The distribution of the presumed DA innervations outside the incertohypothalamic fiber system is summarized in Fig. 2. In the *paraventricular nucleus* (hvp; level 3) remaining axons formed a terminal plexus with a density about 20–30% of normal (Fig. 5A). The majority of fibers were delicate and sometimes formed basket-like arrangements surrounding non-fluorescent neuronal perikarya. In addition, a minor portion of coarser fibers (most likely NA-containing ones) were found, having a morphology similar to that of the majority of hvp terminals in the normal animal. No obvious differences in the density of the delicate fibers were observed between various subdivisions of the hvp.

A significant plexus of CA fibers also remained in the *supraoptic nucleus* (nso; levels 1–2) after the neurotoxin treatment (Fig. 4B). The axons were mostly delicate and fine-varicose and, similar to the fibers in the hvp, they sometimes had a basket-like arrangement around non-catecholaminergic cell bodies. The remaining fiber plexus was concentrated to the ventral part of the nso where it had a density of about 20–30% of normal. In the dorsal parts of nso, only scattered axons were found.

The *periventricular nucleus* (per; level 2) con-

tained a loose plexus of fibers oriented mainly in the dorso-ventral direction. Some of the fibers were collaterals from the scattered axons crossing the midline in the supraoptic commissures. Others seemed to originate locally in the CA cells of the A14 cell group, which were clearly observable in the per of neurotoxin-treated animals.

In the *dorsomedial nucleus* (ndm; level 4–5) a uniform plexus of fine varicose axons was found and also a minor population of coarser terminal fibers (Fig. 5B). These non-lesioned axons constituted about 20% of the normal innervation. Only scattered CA fibers remained in the posterior hypothalamic nucleus and in the mammillary nuclei.

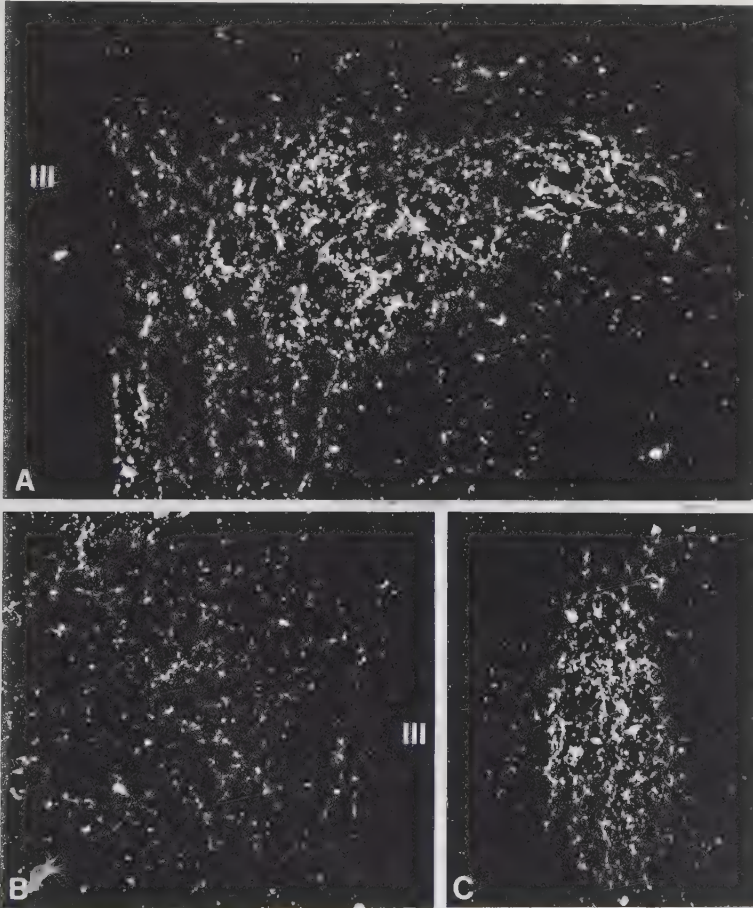


Fig. 5. Remaining CA-containing fibers in frontal sections through (A) the paraventricular hypothalamic nucleus ($\times 200$), (B) the medial part of the dorsomedial hypothalamic nucleus ($\times 140$), and (C) the paraventricular thalamic nucleus ($\times 210$) after the combined 6-OHDA-DSP-4 lesioning procedure. Most axons are delicate and fine-varicose but a minor portion of coarser fibers can also be observed. The DA:NA ratios suggest that a significant part of the terminals are DA-containing. III, third ventricle.

In the *medial zona incerta*, dorsomedial to the hpv there remained an aggregation of fibers belonging to the *zona incerta* bundle of the periventricular system (cf. ref. 24). In addition to the preterminal axons of the nigro-striatal and mesocortical DA bundles, the *lateral hypothalamus-MFB region* contained a low density of delicate axons, which seemed to form a sparse terminal plexus in this area. At the level of the hpv, the remaining fibers were more densely aggregated near the border to the reticular thalamic nucleus.

Thalamus. The combined neurotoxin treatment reduced NA levels by 90 and 96% in the medial and lateral thalamus, respectively. In the lateral thalamus (including the reticular nucleus) no significant remaining terminal plexus was observed. Remaining axons in the medial part, which had a DA:NA ratio of 1.72, were mainly concentrated to the habenula and the paraventricular thalamic nucleus (pv). In the habenula (level 5), a very dense aggregation of fibers was localized in the medial part of the lateral nucleus as described previously^{24,37}. The innervation in the pv (levels 2–5) consisted primarily of delicate axons but also some coarser fibers were observed (Fig. 5C). The density of this terminal plexus amounted to some 20–30% of normal.

To further support the existence of dopaminergic innervations as indicated by the lesion experiments, DA uptake studies were carried out on sagittal sections through the medial diencephalon of non-lesioned, reserpine-treated animals (for details see ref. 20). After incubation in DA in the presence of desipramine, which is a well-known blocker of DA uptake into noradrenergic neurons (for refs. see ref. 20), terminal plexa were observed in the hpv, per, ndm, pv, lateral habenula and medial *zona incerta*. The densities of these innervations were similar to those of the remaining fiber systems in the same nuclei of lesioned animals. No observations were made on the nso.

DISCUSSION

The combined neurotoxin treatment revealed new features of the distribution of DA-containing fibers in the diencephalon. Previous studies have demonstrated two distinct sets of DA-containing fibers in the diencephalon, outside the tuberohypophyseal

system. They are the so-called incertohypothalamic fiber system (distributed in the medial *zona incerta* and the dorsal and anterior hypothalamus)^{5,24}, and the periventricular fiber system which is distributed in the periventricular areas of the diencephalon and the periaqueductal gray of the mesencephalon^{19,24}. Knowledge of the terminal distributions of the periventricular system is still incomplete, however. The principal origin for both these fiber systems is most likely the diencephalic A11, A13 and A14 cell groups.

Apart from the tuberohypophyseal and the incertohypothalamic fiber systems, fluorescent fibers remained only in a few diencephalic nuclei in the neurotoxin-treated animals, above all in the supraoptic, paraventricular and dorsomedial hypothalamic nuclei, the paraventricular thalamic nucleus and the lateral habenula. The DA:NA ratios in the lesioned animals indicate that a majority of these fibers were DA-containing, and this conclusion is also supported by several other biochemical and microscopic data. (a) Determinations of CAs, also in normal animals, signify that DA in these nuclei is not only a precursor of NA, but plays an independent transmitter role in separate DA terminal systems. Thus, in untreated rats, high DA:NA ratios have been found in the supraoptic (0.16–0.20), paraventricular (0.16–0.20) and dorsomedial (0.11–0.39) hypothalamic nuclei, in the habenula (0.51) and in midline thalamic nuclei, including the paraventricular thalamic nucleus (0.34)^{30,40}. This should be compared to the DA:NA ratio, e.g. in the anteroventral thalamic nucleus (0.09), the CA innervation of which is considered to be exclusively noradrenergic. (b) Tyrosine hydroxylase (TH) immunohistochemistry which preferentially, although not exclusively, demonstrates DA neurons, has shown TH-positive terminal systems in those hypothalamic, thalamic and epithalamic nuclei which contained fiber plexa after combined neurotoxin treatment¹⁵. (c) Observations on DA uptake into Vibratome sections from the diencephalon in the present study demonstrated fiber systems with a desipramine-resistant uptake of CAs in these same nuclei. Since desipramine is known to block the uptake into noradrenergic neurons but not into dopaminergic ones, these findings support the existence of dopaminergic innervations as indicated by the lesion experiments. (d) Buijs and co-workers

(ref. 8 and personal communication) have recently been able to demonstrate immunohistochemically a DA-containing fiber plexus in the paraventricular, supraoptic and dorsomedial hypothalamic nuclei as well as in the paraventricular thalamic nucleus and the lateral habenula, similar to that reported in the present study, using a specific antibody against DA.

It is now well established that DA neurons located in the ventral tegmental area (VTA) project to the lateral habenula^{2,14,31,32,36,37}. The origins of other presumed dopaminergic innervations in the diencephalon demonstrated in this study are, however, largely unknown. Evidence is contradictory regarding the possible contribution of the mesencephalic DA cell groups. After injections of anterograde tracers into the VTA, Simon and coworkers³⁶ found labeling in the hypothalamus, including the dorsomedial and supraoptic nuclei and, to a lesser degree, the paraventricular nucleus. There are also some biochemical data to support a contribution from these cells to the DA innervation of the paraventricular thalamic nucleus¹⁸. In contrast, other studies using both anterograde and retrograde tracers have not succeeded in corroborating the existence of projections from the DA cells in VTA or substantia nigra to these hypothalamic nuclei^{2,3,11}. Presently available experimental data thus indicate that the VTA and substantia nigra DA cells have, at most, minor projections to the thalamus and hypothalamus.

The A13 and A14 cell groups in the anterior and dorsal hypothalamus, or the A11 cells within the periventricular DA system are, therefore, the most likely sources for the diencephalic DA innervations. The DA-containing cell bodies of this system are distributed in the caudal thalamus and along the periaqueductal gray and extend into the dorsal raphe nucleus. It is interesting in this context that injections of horseradish peroxidase into the paraventricular hypothalamic nucleus have been reported to label neurons both in the periaqueductal gray and in the dorsal raphe³⁸. The transmitter content of these labeled cells was not identified and the possible DA nature of the connections therefore remains to be established. The new, transmitter-specific retrograde tracing techniques¹⁶ should be ideal for solving this problem.

The present demonstration of direct DA-containing fiber supplies to the paraventricular and supraoptic nuclei provides further support for the idea that the dopaminergic control of oxytocin and vasopressin release is exerted, at least partly, at the cell body level. At the hypothalamic level, DA seems to stimulate the release of both oxytocin and vasopressin. Moss and co-workers²⁹ and Mason²⁷ have provided evidence that this excitatory effect of DA can be elicited by direct application of DA onto the neurosecretory cell bodies, *in vivo* or *in vitro*. Moos and Richard³⁸ have suggested that hypothalamic DA normally acts to facilitate the reflex release of oxytocin (induced by suckling) and regulates the release of vasopressin in response to osmotic challenges.

The anatomical arrangement of the diencephalic DA neurons provides possibilities for DA to exert effects on the hypothalamic neurosecretory neurons both at the cell body level and at the level of the terminals in the neural lobe of the pituitary. DA neurons in the rostral part of the arcuate nucleus have thus been shown to project to the neural lobe^{4,6}, where they make synaptoid contacts with the terminals of the neurosecretory neurons¹. Consistent with this, DA has been shown to have modulatory effects on vasopressin and oxytocin release also from isolated neural lobes *in vitro*^{7,35,41}.

Available data thus suggest that DA participates in the complex regulation of hormone secretion from the magnocellular neurosecretory neurons both through the elements of the tuberohypophyseal DA system innervating the neural lobe, and through diencephalic DA neurons innervating the supraoptic and paraventricular nuclei. Further studies are now in progress to identify the cell bodies of origin for the latter innervations.

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ORIGIN, COURSE AND TERMINATION OF THE MESO-
HABENULAR DOPAMINE PATHWAY IN THE RAT

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Origin, Course and Termination of the Mesohabenular Dopamine Pathway in the Rat

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This study describes the organization of the mesohabenular dopamine (DA) system in the rat as revealed by fluorescence histochemistry in combination with lesions, DA uptake experiments and injections of a retrograde tracer. The DA axons were found to be aggregated in a dense terminal field located in the caudal two thirds of the medial part of the lateral habenular nucleus. Microknife lesions of the stria medullaris left this DA innervation unaffected while cuts through the fasciculus retroflexus resulted in the virtual disappearance of the DA innervation. Injections of the fluorescent retrograde tracer True Blue (TB) into the lateral habenula produced labeling of both DA and non-DA-containing cells in the ventral mesencephalon, mainly in the interfascicular nucleus ipsilateral to the injection. This study thus documents the existence of a mesohabenular DA pathway whose cell bodies are located in the ventral mesencephalon and whose axons ascend with the fasciculus retroflexus to terminate in the caudomedial part of the lateral habenular nucleus. This information, taken together with insights gained from other studies, suggests a role for the mesohabenular DA system in modulating telencephalic feedback onto the mesencephalic DA-neurons and also in regulating the output from the dorsal raphe nucleus.

INTRODUCTION

The interest in the mesencephalic dopaminergic neuron system has classically been focused on its heavy projections to striatal and cortical forebrain areas. These neurons are, however, known to also innervate areas in the lower parts of the neuraxis, i.e. diencephalic and pontine regions (see, refs. 6,7,25 for reviews). One such target area for dopaminergic afferents of mesencephalic origin is the habenula. A dense DA innervation was first described by Lindvall et al.²⁵ in the medial part of the lateral habenular nucleus, and this has later been confirmed by Hökfelt et al.¹⁷ and by Phillipson and Pycoc³⁷. There is substantial evidence from lesion studies, as well as from experiments with anterograde and retrograde tracers that this DA input originates in the A10 cell group situated in the ventromedial mesencephalic tegmentum^{5,15,21,36,39,42}.

The present study was undertaken to delineate more precisely the localization of the cells of origin of

the mesohabenular projection system and to map the pathway and innervation territory of the dopaminergic mesohabenular axons.

MATERIALS AND METHODS

The study was performed on young adult female Sprague–Dawley rats.

Histochemistry

Catecholamine (CA)-containing neurons were visualized with the aluminum-catalyzed formaldehyde (ALFA) method for freeze-dried, paraffin-embedded specimens (Lorén et al.²⁹, Procedure I). In brief, the rats were perfused, via the ascending aorta, first with ice-cold Tyrode's buffer (pH 7.0) and then with ice-cold paraformaldehyde in Tyrode's buffer, containing a high concentration of $Al_2(SO_4)_3$. The brains were quickly frozen in liquid propane, cooled to between -70 and -90 °C with liquid nitrogen, and then freeze-dried, reacted with formaldehyde

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vapor (+80 °C for 1 h) and embedded in paraffin in vacuo. Serial 15 µm sections were cut in the frontal and sagittal plane.

Neurotoxin pretreatment

For visualization of DA-containing axons in the diencephalon, in the virtual absence of interfering noradrenaline (NA) fluorescence, some rats were pretreated with 6-hydroxydopamine (6-OHDA) injected bilaterally into the ascending NA fiber bundles in the caudal mesencephalon, followed 1 and 2 weeks later by subcutaneous injections of the selective NA neurotoxin DSP-4 (2 × 50 mg/kg s.c., 1 week apart). For details of this pretreatment and its biochemical effects, see Lindvall et al.²⁶

Dopamine uptake experiments

For differentiation between dopaminergic and noradrenergic structures we also used vibratome sections from fresh brain tissue of normal rats. The sections were incubated according to the method described previously²⁴ in 5×10^{-6} M DA for 20 min, with or without desipramine (10^{-5} M; Ciba-Geigy). The incubations were performed on sections from animals treated with reserpine (Ciba-Geigy, 5 mg/kg, 12–18 h before sacrifice) and nialamide (Pfizer; 300 mg/kg, 1–3 h before sacrifice). The sections were then processed according to the glyoxylic acid method²⁴ and examined by fluorescence microscopy. The uptake of DA into dopaminergic fibers is unaffected by desipramine, whereas that into noradrenergic fibers is blocked. The fluorescent fibers that can be observed in the specimens incubated with desipramine are therefore only the dopaminergic ones.

Tracer injections

Combined fluorescent retrograde tracing and aldehyde fluorescence histochemistry was performed according to Björklund and Skagerberg⁹ using True Blue (TB) as the tracer compound. 20–80 nl of a 3% sonicated suspension of TB was injected into the lateral habenula, or adjacent control regions. The tracer was delivered during 5–10 min through a glass capillary attached to a 1 µl Hamilton syringe equipped with a microdrive. After a survival time of 5–12 days the animals were perfused, under chloral hydrate anesthesia, with an ice-cold buffered ALFA solution (pH 5.0), and processed as above.

Mechanical lesions

The stria medullaris and the fasciculus retroflexus were transected unilaterally by means of a micro-knife with a 2 mm horizontal cutting edge, as illustrated in Fig. 4. The stria medullaris lesion was placed 2–2.5 mm behind bregma (6 mm down). The fasciculus retroflexus lesion was made at a 35° angle to the vertical plane with the tooth bar set at about –11.5 mm, which was the lowest possible level on the stereotaxic instrument used (Kopf model 900). The knife was advanced 7.5 mm from the brain surface in the rostroventral direction. The rats were processed for CA histochemistry 2–3 weeks after lesion.

Fluorescence microscopy

In the ALFA method, the DA-derived fluorescence was visualized at 405–435 nm excitation (Schott BG12 as lamp filter). For combined visualization of TB and DA the activating light was switched between 435 nm (for visualization of DA alone) and 365 nm (for visualization of TB and DA). For details of this combined procedure, see Björklund and Skagerberg⁹ and Hökfelt et al.¹⁸

RESULTS

Terminal distribution

In the habenula of the normal animal the catecholamine (CA) fibers are aggregated in the medial part of the lateral nucleus and it has been suggested that a major portion of these terminals are DA-containing (cf. above). In the present study we have used two experimental approaches to visualize selectively the DA innervation of the habenula. First, a combination of intracerebral 6-OHDA injections in the caudal mesencephalon with systemic administration of DSP-4, a procedure which causes an extensive depletion of forebrain NA but leaves DA systems virtually unaffected²⁶. Second, DA-uptake studies were performed on sections, prepared from normal reserpine-treated rats, in the presence or absence of desipramine in the incubation medium. With desipramine present the DA uptake into NA fibers is blocked, which allows for selective visualization of DA fibers. The distribution of the DA innervation in the habenula, as revealed by the DA uptake experiments, and the combined 6-OHDA + DSP-4 lesions, is summa-

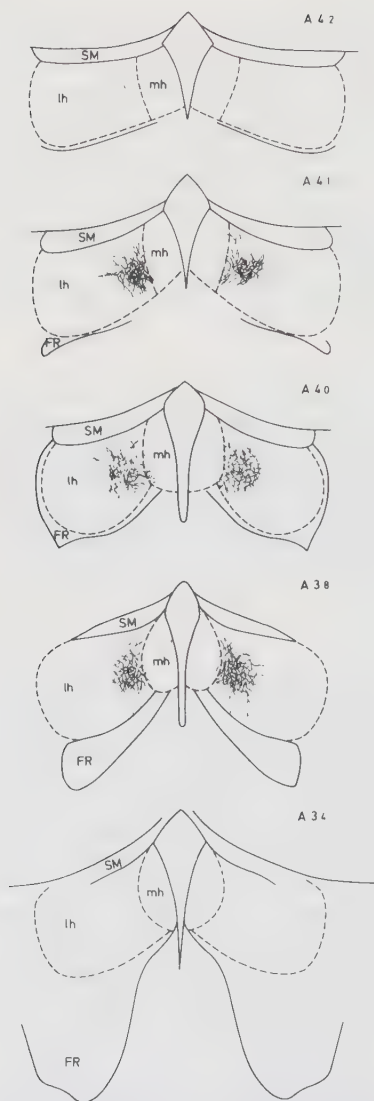


Fig. 1. Semischematic representation of the distribution of DA fibers in the habenula at 5 coronal levels through the rat brain. With the exception of some very few fibers in the medial habenula the DA fibers are restricted to the medial part of the later-

alized in 5 closely spaced coronal levels in Fig. 1. The fibers were aggregated in the medial part of the lateral habenular nucleus, with occasional threads penetrating into the most lateral portion of the medial nucleus. In the mediolateral dimension the fiber aggregation extended from the border between the medial and lateral nuclei about 0.2–0.3 mm laterally. In the anteroposterior dimension the innervation covered the caudal two-thirds of the nucleus, from close to the posterior end (about level A 3.4 mm in the atlas of König and Klippel²², see Fig. 1) and 0.5–0.6 mm rostrally. Other parts of the lateral nucleus contained only scattered fibers. In the neurotoxin-treated animals a minor plexus of fluorescent fibers remained in the medial habenula, particularly in its dorsomedial part. Most of these remaining fibers could, on basis of their fluorescence morphology⁸, readily be identified as noradrenergic axons of sympathetic origin, but some very few delicate fibers with low fluorescence intensity was in some cases seen deep in the medial nucleus.

True Blue injections

After injections of the retrograde tracer TB into the caudal half of the lateral habenula (Fig. 3B) retrogradely labeled cells were found in several regions in the forebrain and brainstem. Retrogradely labeled DA-containing cell bodies were, however, found exclusively in the ventral tegmental area (VTA) in the mesencephalon; therefore, the distribution of TB-labeled cell bodies was studied in detail only in this region. As illustrated in Fig. 2, the majority of retrogradely labeled DA cells were situated close to the midline medial to the ventral tip of the fasciculus retroflexus and just dorsal to the interpeduncular fossa or, more caudally, dorsal to the interpeduncular nucleus. The labeled cells were small, round and usually exhibited a fairly weak DA-fluorescence and were accordingly in all respects typical of cells belonging to the interfascicular nucleus, as described by Phillipson³⁵. A smaller number of TB-labeled dopaminergic cell bodies were found more laterally in the VTA. These cells were medium-sized and showed a more intense DA-fluorescence.

al habenula. Numbers to the right indicate the distance in mm anterior to the ear-bars according to the atlas of König and Klippel²². FR, fasciculus retroflexus; lh, lateral habenular nucleus; mh, medial habenular nucleus; SM, stria medullaris.

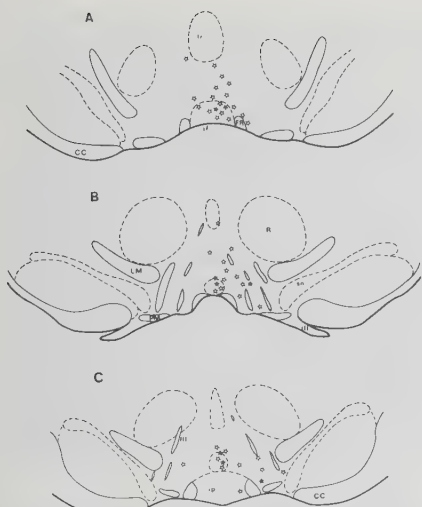


Fig. 2. Schematic representation of the location of cell bodies that were retrogradely labeled after an injection of TB into the lateral habenula (injection site illustrated in Fig. 3B). The sections represented spans the rostrocaudal extent over which labeled DA cells were found. Each filled star denotes one labeled cell in one 15 μ m thick section. Retrograde labeling is strongly ipsilateral with most of the labeled DA cells found in the inter-fascicular nucleus while many of the non-DA TB labeled cells (open stars) occurs both lateral and dorsal to this nucleus. Note that the sections have been chosen especially to show retrogradely labeled DA cells whose total number may accordingly seem exaggerated. CC, crus cerebri; FR, fasciculus retroflexus; if, interpeduncular fossa; ip, interpeduncular nucleus; LM, medial lemniscus; PM, mamillary peduncle; R, red nucleus; sn, substantia nigra, III, root fibers of the oculomotor nerve.

Throughout the rostrocaudal levels studied, the majority of retrogradely labeled neurons did not exhibit DA-fluorescence. In fact, within the restricted region of the interfascicular nucleus, where most of the retrogradely labeled DA cells were found, the DA cells constituted no more than 20–30% of the total population of retrogradely labeled cells. Outside this nucleus only some 5–10% of the labeled cells were DA-containing. As mentioned above both DA and non-DA cells were labeled in the ventral part of the VTA while almost only non-DA neurons were found to be labeled in more dorsal areas.

In 3 animals the total number of tracer labeled cells, through the rostrocaudal extent where labeled DA cells were found, was estimated by counting the

cells in the microscope and applying Abercrombie's correction formula¹. A total of 50–70 TB-labeled cells were found throughout the mesencephalic levels studied, and of these between 5 and 15 were DA-containing. About 2/3 of these dopaminergic cells were found within the confines of the interfascicular nucleus. Of all retrogradely labeled cells more than 90% were found ipsilateral to the injection site; most of the cells found contralaterally were non-DA-containing.

Since in all cases some leakage of tracer occurred along the injection track, control injections were made where the tracer was deposited either in the dorsal hippocampus (Fig. 3A) or in the overlying neocortex. Control injections were also made in the dorsal thalamus rostral and caudal to the heavily innervated part of the lateral habenula (Fig. 3A, C). All these control injections failed to label any DA cells in the mesencephalon, but it is notable that the injection that involved the rostral part of the lateral habenula (Fig. 3A) labeled a significant number of non-DA cells in the VTA.

Microknife lesions

Since it is known that both the stria medullaris and the fasciculus retroflexus contains CA axons²⁷ and anterograde tracer studies have suggested that neurons in the VTA may innervate the habenula via both fiber-tracts we decided to lesion these inputs separately. Transection of the stria medullaris (rostral cut in Fig. 4) caused a pile up of CA fluorescence both rostral and caudal to the cut, but there was no clear-cut effect on the habenular DA innervation. By contrast, unilateral transection of the fasciculus retroflexus (caudal cut in Fig. 4) removed a major portion of the DA fibers in the lateral habenula ipsilateral to the lesion (Fig. 5). The denervating effect was estimated to be in the order of 70–95%. Pile-up of catecholamine fluorescence occurred ventral to the cut in fibers ascending along the fasciculus retroflexus towards the habenula (Fig. 6). These fibers occurred both within the fasciculus as well as on its medial and lateral surfaces.

DISCUSSION

The present results provide evidence (i) that the habenular DA innervation originates in cells in the

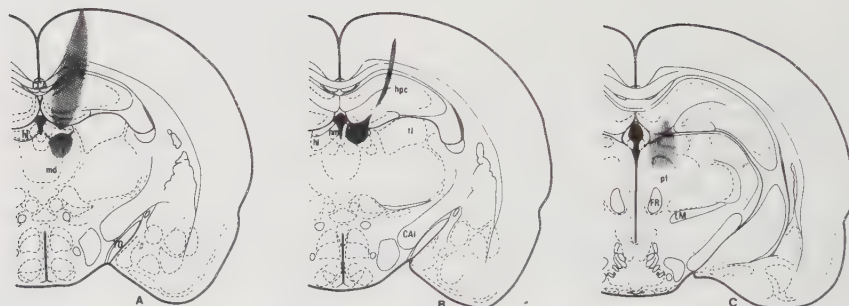


Fig. 3. The location of some tracer injections at 3 coronal levels. Stippled areas in A and C delineates the maximal extension of control injections rostral (dorsal and ventral) and caudal to the area of DA innervation, which did not label any DA cells. In B the black area shows the injection site resulting in the pattern of retrograde labeling illustrated in Fig. 2.

VTA, located primarily within the so-called interfascicular subnucleus; (ii) that the axons ascend, probably exclusively, along the fasciculus retroflexus; (iii) that the habenular DA innervation is confined to the caudal two-thirds of the medial part of the lateral nucleus; and (iv) that the projection is almost completely uncrossed. These principal features of the mesohabenular DA projection are summarized schematically in Fig. 7.

An important conclusion from the present study is that the DA neurons constitute only a minor portion of the VTA projection to the habenula. Thus, maximally some 10% of the total labeled cell population in the VTA was DA-containing, and even within the interfascicular subnucleus, where the majority of the

mesohabenular DA cell bodies were found, the DA-containing neurons constituted no more than 20–30% of the total population. The features of the DA and non-DA mesohabenular neurons are however, in several ways different: (i) while the DA neu-

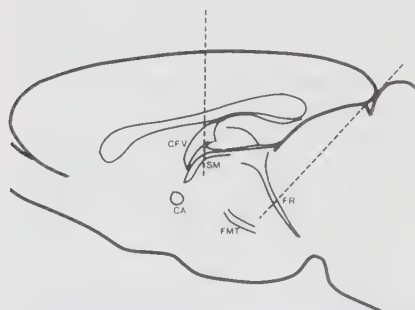


Fig. 4. Location of knife cuts (dashed lines) used to sever the stria medullaris or fasciculus retroflexus. CA, commissura anterior; CF, commissura fornicis ventralis; FMT, fasciculus mamillothalamicus; FR, fasciculus retroflexus; SM, stria medullaris.

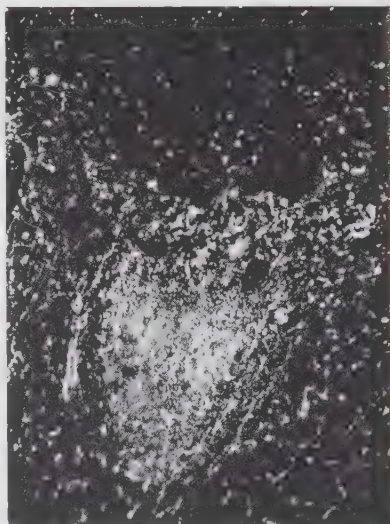


Fig. 5. Photomicrograph showing a knife-cut through the fasciculus retroflexus and the pile-up of fluorescent fibers in and along the fiber tract. Frontal section, $\times 125$. Publisher's reduction factor 0.8.

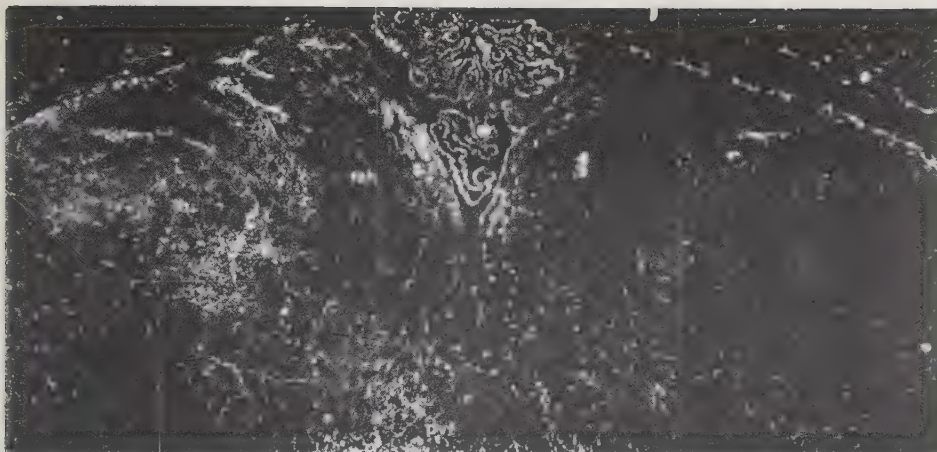


Fig. 6. This photomontage illustrates the effect of a unilateral fasciculus retroflexus lesion on the appearance of monoamine fluorescence in the habenula. To the left is seen the normal distribution of fluorescent fibers with the aggregation of DA-fibers in the medial part of the lateral habenula. To the right is seen the almost complete disappearance of this aggregation after a knife cut through the ipsilateral fasciculus retroflexus. Most of the fibers in the medial habenula are of sympathetic origin; that few such fibers are seen on the right side should not be interpreted as an effect of the lesion but reflects the normally occurring uneven distribution of these fibers. mh, medial habenular nucleus; lh, lateral habenular nucleus; pvt, paraventricular thalamic nucleus. Frontal section. $\times 84$. Publisher's reduction factor 0.6.

rons were largely confined to the interfascicular nucleus, the non-DA neurons were more widely dispersed in the VTA (cf. Fig. 2); (ii) the non-DA projection seemed to have a larger crossed component; (iii) while the DA innervation was restricted to the caudal part of the medial lateral habenula, the tracer results suggested that the non-DA projection has a wider distribution throughout the habenula.

The results presented here are in accordance with earlier studies using HRP as the retrograde tracer and showing a projection from the medial VTA to the lateral habenula^{14,36} and also confirms the suggestion that this projection, at least partly, is dopaminergic in nature³⁶. While our results show that, though being a minority of the retrogradely labeled cells, the dopaminergic neurons constitute a substantial portion of the mesohabenular pathway, a recent study⁴² has, however, claimed that this system is almost entirely non-dopaminergic. With the present results in hand this apparent discrepancy can be resolved by considering the restricted distribution of DA terminals in the lateral habenula. Thus, since the DA innervation

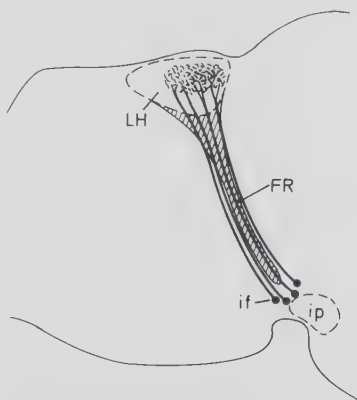


Fig. 7. Schematic representation of the salient features of the mesohabenular DA-system. The cell bodies are located in and close to the interfascicular nucleus (if) just rostral or dorsal to the interpeduncular nucleus (ip). The axons ascend with the fasciculus retroflexus (FR) and terminate in the caudal half of the lateral habenula (lh).

is confined to the medial and caudal parts of the lateral habenula, small injections rostral or just lateral to the DA terminal field (as the injection site illustrated by Swanson⁴²) would not be expected to label DA cell bodies. Our finding that the (dopaminergic and non-dopaminergic) mesohabenular pathway is to at least 90% uncrossed is also supported by other studies^{4,36,42} but may seem not to be fully in accord with our lesion experiments which, judged by subjective estimations of fluorescence intensity, pointed to a slightly lower degree of denervation. Since, however, the lesion experiments were performed on animals with intact NA innervation, the remaining fluorescence after the knife cuts through the fasciculus retroflexus may be largely contributed to by the NA system⁴³, probably ascending to the habenula via the stria medullaris.

It is notable that in the 3 animals in which cell-counts were performed after unilaterally placed TB injections the number of labeled DA neurons in the VTA never exceeded 15. This suggests that within the mesencephalic dopaminergic projection system the innervation of a restricted target territory (such as the lateral habenular nucleus) can be derived from a very low number of neurons. This is consistent with estimates based on quantitative biochemical measurements. The average total DA content of single DA neurons in the rat brain has been estimated at approximately 30 pg^{6,7}. Since the total DA content of the habenula on one side probably is in the range of 200–500 pg*, only some 10–20 DA neurons would be necessary to account for the total habenular DA innervation. By comparison (see ref. 7) the entire telencephalon on one side (striatal, limbic and cortical regions combined) contains some 550,000 pg of DA and is innervated by 15,000–20,000 neurons in the mesencephalic DA cell group. The small number of mesohabenular neurons can be taken to illustrate the high degree of anatomical specialization that exists in this DA projection system, a principle that may be valid also for other neuronal subsets of the mesencephalic DA neuron system.

The interfascicular nucleus has been identified as a subgroup of the mesencephalic DA neuron system

mainly on cytoarchitectural grounds^{36,42}. This subgroup is, however, not homogeneous with respect to its projections. While it is the principal source for the mesohabenular projection, other cells in this subgroup have been shown to project also to n. accumbens, lateral septal nucleus, anterior cingulate cortex, amygdala, entorhinal cortex and locus coeruleus⁴². Swanson's⁴² fluorescent tracer study indicates, however, that with some possible single exceptions, the neurons projecting to the habenula (both the DA and the non-DA-containing ones) have no collateral projections to the other target areas.

As shown in Fig. 1 the dopaminergic fibers in the habenula are characteristically confined to the medial part of the lateral nucleus; the few fibers that penetrate through the border to the medial nucleus are conceivably also functionally related to the lateral nucleus since it has been shown that dendrites of cells located in the lateral habenula may for short distances reach into the medial nucleus¹⁹. We believe that this medial portion of the dopaminergic fiber aggregation is the most likely explanation for the rather high levels of DA that have been reported to exist in the medial nucleus³⁷. It seems unlikely that the few delicate fibers we sometimes observed in the medial habenula could account for the DA levels measured in this nucleus since they amount to only a tiny fraction of the fluorescent fibers seen in the microscope.

The potential functional role of the mesohabenular projection gains its interest from the importance of the lateral habenula as a relay station for descending output pathways from limbic, hypothalamic and striatal forebrain regions. As summarized schematically in Fig. 8, the lateral habenula receives its principal afferents from the entopeduncular nucleus (which is a classical link for the efferents from the caudate-putamen) and the lateral hypothalamus, as well as from several areas of the limbic system and the ventral striatal complex, such as the ventral pallidum (including the substantia innominata and the lateral preoptic area), the n. accumbens and the prefrontal cortex^{5,14,15,33,41}. Of particular interest is the recent demonstration of Greatrex and Phillipson¹⁴, in the rat, that the projection from the anteromedial

* Assuming that the volume of the rat habenula is in the order of 1.5–1 mm³, the concentration figures for DA given by Versteeg et al.⁴⁴ and Phillipson and Pycock³⁷ (averaging about 5 pg/ μ g protein for medial and lateral habenula combined) give an approximate total DA content of 200–500 pg.

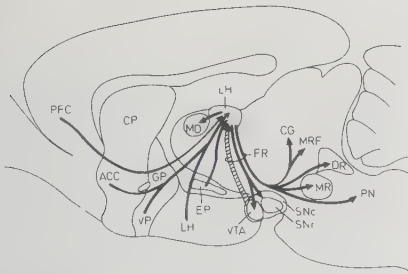


Fig. 8. Highly schematized figure in the sagittal plane through the rat brain which illustrates some of the afferent and efferent connections of the lateral habenular nucleus. ACC, nucleus accumbens; CG, central grey; CP, caudatus-putamen; DR, dorsal raphe nucleus; EP, entopeduncular nucleus; FR, fasciculus retroflexus; GP, globus pallidus; LH, lateral hypothalamus or lateral habenula; MD, mediodorsal thalamic nucleus; MR, median raphe nucleus; MRF, mesencephalic reticular formation; PFC, prefrontal cortex; PN, nuclei in the pons; SNc, substantia nigra-pars compacta; SNr, substantia nigra-pars reticulata; VP, ventral pallidum.

frontal cortex terminates in the same part of the lateral habenula as the mesohabenular DA pathway.

The principal efferent pathways of the lateral habenula includes projections to the mediodorsal thalamic nucleus (which in turn is the principal thalamic source for afferents to the anteromedial frontal cortex)^{3,16} and to several mesencephalic and pontine regions, above all the dorsal and median raphe nuclei, the mesencephalic reticular formation and the central gray^{2,16,34,41}. These latter projections provide routes for influences both onto descending brainstem pathways and onto the ascending serotonergic raphe projections. Lateral habenula also has efferents to the pars compacta of the substantia nigra and the VTA, i.e. the regions of the mesencephalic DA neurons. In fact, the area of the lateral habenula innervated by the mesohabenular DA pathway appears to overlap with those parts of the nucleus which contain the cell bodies of origin for the projection to the dorsal raphe nucleus³⁴ and to the substantia nigra VTA region¹⁶. This points to a potential role of the lateral habenula in feed-back control or modulation of the activity of the mesotelencephalic DA projection system. There is already some evidence for this, since lesions of the habenula have been reported to cause activation of the DA neurons projecting to the frontal cortex²⁸. Furthermore, Christophe et al.¹² have recently reported that electrical stimulation of

the lateral habenula caused inhibition of as much as 95% of all identified DA neurons in the VTA and 85% of all identified DA neurons in the substantia nigra. This supports the previous anatomical finding¹⁶ that the efferent projection from the lateral habenula has a widespread area of termination in the ventral mesencephalon.

The role of DA in habenula can at this stage only be speculated on. It is in this context interesting that studies with the autoradiographic deoxyglucose method have shown marked changes in the functional activity of the lateral habenula after manipulation of dopaminergic mechanisms. Systemic administration of apomorphine^{30,31} and amphetamine⁴⁵ decreases glucose utilization in the lateral habenula and haloperidol³² and lesions of the entire ascending system from the mesencephalic DA cells^{23,38,46} increase glucose utilization in the lateral habenula. These results indicate not only that stimulation of DA receptors somewhere in the brain inhibits glucose use but also that dopaminergic mechanisms directly or indirectly exert a depressant resting tone on glucose consumption in the lateral habenula.

Can the changes in glucose use in the lateral habenula after drugs or lesions be explained by interference with the function of the mesohabenular DA system? In this context it is important to remember that the lateral habenula has direct or indirect afferent connections with several areas known to have rich DA inputs such as the anteromedial frontal cortex and the caudate-putamen. The changes in habenular glucose consumption might thus be secondary to manipulations of DA mechanisms in these areas, mediated via non-dopaminergic systems to the lateral habenula. Several data now indicate that, in fact, the habenular changes might be referred primarily to alterations in the caudate-putamen and mediated via the entopeduncular nucleus. First, the increase of glucose utilization in the lateral habenula after DA bundle lesions is reversed by apomorphine injections into the striatum²³. Second, kainic acid-induced lesions of the striatum reproduce the effects of DA bundle lesions and increase glucose utilization in the lateral habenula²⁰. Third, intrastratial DA infusion leads to a reduction of glucose use in the lateral habenula¹¹ and this mimics the action of systemic apomorphine. The present study provides further evidence that the mesohabenular DA system is not involved in

the changes in habenular glucose use induced by lesions or systemic dopaminergic drugs. Thus, these manipulations cause alterations in glucose use mainly in the lateral part of the lateral habenular nucleus^{23,46} whereas the mesencephalic dopaminergic afferents distribute almost exclusively to its medial part.

The anatomical data suggest that the habenular DA innervation is favorably situated to regulate the descending influences from limbic and striatal forebrain areas onto, above all, the substantia nigra-VTA region, mesencephalic reticular formation, and the raphe nuclei. Such a functional role for the mesohabenular DA pathway finds support in the recent results by Glowinski and Soubrié¹³ showing that DA infused directly into the lateral habenula in cats reduces [³H]serotonin release (labeled from [³H]tryptophan) in substantia nigra bilaterally. This effect was blocked by DA receptor antagonists. A similar effect on nigral [³H]serotonin release was obtained after electrical stimulation of the lateral habenula and in both cases the effects were blocked by direct application of the GABA blocker, picrotoxin, to the dorsal raphe nucleus. This suggests that the DA effect was mediated via an action on the GABAergic lateral habenula-dorsal raphe connection (see Fig. 8)

resulting in an inhibition of the serotonergic neurons of the dorsal raphe innervating the substantia nigra. Taken together, available data indicate that the mesohabenular DA pathway participates in the control of habenular feed-back circuits regulating dopaminergic neurotransmission in striatal and cortical forebrain areas, as well as in the interplay between the dopaminergic and serotonergic systems in the brain. In the striatum, the mesotelencephalic DA neurons have been proposed to act as a 'level setting' or 'permissive' system where DA may tonically regulate the responsiveness of the striatal neurons to other afferent inputs^{7,10,40}. This may be a valid assumption also for the habenular DA afferents.

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TOPOGRAPHIC PRINCIPLES IN THE SPINAL PROJECTIONS
OF SEROTONERGIC AND NON-SEROTONERGIC
BRAINSTEM NEURONS IN THE RAT

G. Skagerberg and A. Björklund

Abstract

The spinal projections from the raphe-associated brainstem areas containing serotonergic neurons were studied with aldehydeinduced fluorescence in combination with the retrograde fluorescent tracer True Blue in the rat. This technique makes it possible to simultaneously determine the projections of individual neurons and to detect whether serotonin is present in the same neurons. After tracer injections into the spinal cord retrogradely labeled serotonergic and non-serotonergic neurons were found in the medullary raphe nuclei and adjacent regions and to a lesser extent in association with the dorsal and median raphe nuclei in the mesencephalon. Large TB injections that covered one side of the spinal cord at mid-cervical level labeled about 60% at the ipsilaterally situated serotonergic neurons in the medullary raphe regions while the corresponding figure contralaterally was about 25%. On both sides a larger number of labeled non-serotonergic neurons were found; they were sometimes located dorsal, but often intermingled with, the serotonergic cells.

While the serotonergic projection from the mesencephalon could not be labeled from injections below cervical levels, the labeling in more caudal brainstem regions exhibited only minor variations depending on rostro-caudal level of the spinal segment injected. Furthermore, quantitative data from injections at different levels indicate that the majority of the spinal-projecting neurons traverse most of the length of the cord.

Summarizing the results obtained from small injections restricted to subregions of the cord we have distinguished three fairly distinct pathways for spinal projections from the medullary raphe and adjacent regions: 1. The dorsal pathway originates mainly from cells in the caudal pons and rostral medulla oblongata (rostral part of nc. raphe magnus, nc. raphe magnus proper, nc. reticularis gigantocellularis pars α , and nc. paragigantocellularis). This pathway, which contains a large non-serotonergic component, descends through the dorsal part of the lateral funiculus and terminates mainly in the dorsal horn at all spinal cord levels. 2. The intermediate pathway is largely serotonergic with its cellbodies located within the accurate cell group (situated just ventral and lateral to the pyramids very close to the ventral surface of the brainstem) and in the nc. raphe obscurus and pallidus, and terminates in the intermediate grey at thoraco-lumbar and upper sacral levels.

3. The ventral pathway consists of serotonergic and non-serotonergic axons descending in the ventral white matter to terminate in ventral horn areas along the entire length of the cord. These axons originate mainly from neurons in the caudal medulla (nc. raphe obscurus and pallidus and nc. reticularis ventralis).

This study shows that from all brainstem regions which give rise to spinal serotonin projections there also exists (often larger) non-serotonergic spinal projections. While the serotonergic systems are highly topographically arranged the non-serotonergic projection (taken as a whole) is more diffusely arranged.

Abbreviations: CA, catecholamine; DLF, dorsal part of the lateral funiculus; 5HT, 5-hydroxytryptamine; IML, intermediolateral cell column; MAO, monoamineoxidase; NIH, nucleus interfascicularis hypoglossi; PGCL, nucleus paragigantocellularis lateralis; RGC, nucleus reticularis gigantocellularis; RGC α , nucleus reticularis gigantocellularis pars α ; RM, nucleus raphe magnus; RMr, rostral extension of nucleus raphe magnus; RO, nucleus raphe obscurus; RP, nucleus raphe pallidus; RPC, nucleus reticularis pontis caudalis; RV, nucleus reticularis ventralis.

Introduction

Spinal projections from the medullary raphe nuclei, i.e. *nc. raphe magnus* (RM), *raphe obscurus* (RO) and *raphe pallidus* (RP) were described already in 1960 by Brodal et al.¹⁸, although at this time the functional implications of these projections were obscure. The original observation by Dahlström and Fuxe (in 1964)²⁵ that most of the serotonin (5HT)-containing neurons are located within or close to the raphe nuclei gave new impetus to the study of the spinal projections from these nuclei, especially since earlier studies had documented the presence of this amine in the spinal cord^{5,6} and also indicated that it was localized in axons of supraspinal origin²². In their study on the distribution of monoamines in the spinal cord, Dahlström and Fuxe²⁶ described a system of 5HT-containing axons that descended through the peripheral parts of the ventral and lateral funiculi. These axons gave rise to terminals that could be seen in most areas of the grey matter but were apparently concentrated to the regions containing somatic motoneurons in the ventral horn, the intermediolateral (IML) cell column and the superficial layers of the dorsal horn. Consequently, these authors speculated on the involvement of the descending serotonergic system in somatic and autonomic motor function and also in influencing the reflex response to sensory input.

More recent investigations have confirmed that descending 5HT-systems exert an important influence on autonomic functions^{20,36} while other studies indicate a modulatory effect of 5HT on somatic motoneurons^{4,53}.

Most recent attention, however, has been focused on the raphe spinal system as an anatomical substrate for anti-nociceptive mechanisms. This interest was triggered by the discovery that an intact raphe spinal 5HT-system seemed essential for mediating the analgesia provoked by systemically administered morphine^{56,66} or intracerebral stimulation³. Subsequent anatomical studies^{8,9,51,67,68} established a spinal projection from RM and nearby ventral part of the gigantocellular reticular formation (RGC) descending through the dorsal part of the lateral funiculus (DLF) and terminating preferentially in lamina I, II and V. More dorsal parts of the RGC and the more caudally situated RP and RO was, on the other hand, found to project through more ventral parts of the white matter to ventral horn regions. Although these studies provided much new important information regarding these descending systems they could not address the topic as to which neurotransmitters were used in the different systems. This has become an increasingly important question since it seems clear that while raphespinal 5HT neurons are importantly involved in the descending systems which mediate analgesia there apparently exists other raphe-spinal systems which confers descending inhibition onto neurons that respond to noxious stimuli but which seemingly are independent on serotonergic neurotransmission^{38,43,69}. Furthermore, descending inhibition of spinothalamic tract neurons can also be elicited from stimulation of ventromedial brainstem sites outside the raphe nuclei. Already Dahlström and Fuxe²⁵ reported that not all neurons in the raphe nuclei were serotonergic and that the 5HT-containing cellbodies were not entirely confined to these nuclei. Additional evidence for the existence of separate populations of raphe-spinal neurons were obtained by measurements of conduction velocities in such neurons^{71,72}. This heterogeneity was further emphasized by the finding that in many neurons in the caudal raphe region 5HT coexisted with substance P in the same cells^{23,34}. A subsequent study of neurons in this area⁴⁰ has added to the chemical complexity of the system by showing that some of

these cells contain thyrotropin releasing hormone (TRH) in addition to 5HT and substance P. In other cells in this area either compound may occur alone or together with one of the other. The important question of the neurotransmitter content of neurons in this area with verified spinal projections has been addressed in the studies by Bowker et al.^{15,16,17} who found a small proportion of the raphe-spinal neurons to be non-serotonergic while about half of the spinal projecting 5HT neurons also contained substance P.

The aim of the present study was to utilize the fluorescent tracer True Blue (TB) in combination with aldehyde induced monoamine fluorescence histochemistry to investigate in more detail the arrangement of spinal projections from cell groups which contain 5HT cells. By injecting small amounts of TB into restricted regions of the spinal cord and processing the material for simultaneous 5HT and TB visualization¹³ it was possible to map the localization of neurons that project to or through select regions of the cord and also to quantitatively estimate the involvement of 5HT vs. non-5HT neurons in this projection.

With this technique it has also been possible to obtain information on the topographic principles and the collateral organization of the raphe spinal serotonin system. Preliminary results from this investigation have previously been reported in abstract and review form^{14,60}.

Material and methods

About 80 young adult female Sprague-Dawley rats (170-200 g) were used for this study. For TB injections the rats were deeply anesthetized with Ketalar (50 mg/kg b.w.) - Rompun (10 mg/kg b.w.). Two different approaches were used in order to expose the spinal cord. For cervical injections the animals were mounted in a conventional manner in a stereotaxic frame, whereafter the tail was gently stretched, serving both to suspend the animal, so that respiratory movements did not cause any observable motion of the vertebral column, and at the same time to increase the intervertebral space. The neck muscles were then separated from the underlying vertebrae and the intervertebral ligament cut through, exposing the dorsal surface of the cervical cord. For injections at lower spinal levels a laminectomy approach was used: The animals were loosely secured onto a tabletop by adhesive tape over the extremities. After incision through the shaved skin the dorsal tips of the spinal processes were localized and longitudinal cuts were made along the processes until three or four vertebrae were exposed. The connective tissue between two adjacent vertebrae was then cut through so that the most rostral could be lifted enough to expose the spinal canal, the arcs of this vertebra was cut, the lamina folded forward and temporarily fixed in this position. The dura was then cut with the tip of a fine cannula and in some cases the position of the cord was secured by matressing the space between the dura and the bone with small pieces of gelfoam.

A 2-3% suspension of TB (in water, freshly sonicated) was then injected through a coarse glass capillary (outer diameter approximately 100 μ m; drawn by hand from a glass tube with an inner diameter fitting the original Hamilton cannula) fitted onto a 1 μ l Hamilton syringe with an attached mechanical microdrive. Depending on the volumes injected the injections were called small (up to 0.05 μ l), intermediate (up to 0.10 μ l) or large (up to 0.80 μ l). The small and intermediate injections were deposited in select regions of the cord while the large ones were aimed to completely fill one half of the cord at the level of the injection. In the case of large injections

multiple penetrations were made at the injection site, either with the attached capillary or with the original Hamilton syringe cannula, in a deliberate attempt to sever fibre tracts in order to improve the labeling of fibres of passage. The tracer was delivered during 1 - 2 minutes whereafter the capillary was left in place for at least one minute before being slowly retracted. After the injection was completed the cut vertebra was folded back in place and fixed in this position by suturing the longitudinal muscles on both sides together over the bone. No antibiotics or anesthetics were given postoperatively. During the first two days some degree of limb paralysis was often seen but in almost all animals these abnormalities had disappeared by the time of sacrifice.

After appropriate survival times (about one week for cervical injections, five to six weeks for sacral injections) the animals (now weighing 180-250 g) were given a monoamine oxidase (MAO)-inhibitor (pargyline 200 mg/kg) two to three hours before being anesthetized with chloralhydrate (500 g/kg i.p.), perfused and processed according to the ALFA-method⁴⁷, with the perfusion buffer set at a higher pH. In short, the animals were perfused through the ascending aorta first with 150 ml ice-cold Tyrode's buffer under moderate pressure (0.5 bar) and subsequently with 300 ml ice cold modified ALFA solution (20 g paraformaldehyde and 50 g $\text{Al}_2(\text{SO}_4)_3 \times 16 \text{H}_2\text{O}$ per 1000 ml of Tyrode's buffer, pH finally set at 4.7 by adding potassium acetate) under high pressure (up to 2 bar). The brainstem and part of the spinal cord (containing the injection site) was then removed and quickly frozen to the temperature of liquid nitrogen and freeze-dried for five days. The freeze-dried specimens were finally exposed to formaldehyde vapour, embedded in paraffin and sectioned at 15 μm . Every third section was always taken for fluorescence microscopy and in most cases adjacent sections were taken for Nissl staining. Three rats that did not receive TB injections were processed in the same manner, except that no MAO inhibitor was administered before perfusion. These specimens were used to map the distribution of CA fibres in the area that was investigated.

For fluorescence microscopy Zeiss fluorescence microscopes for transmitted light equipped with a HBO 200 mercury lamp was used. For visualization of monoamines together with TB a Schott UG1 filter was used as primary filter. For selective visualization of monoamines (excluding the TB fluorescence) a Schott AL 435 filter was utilized in combination with a Zeiss 47 barrier filter. The 47 barrier filter was often used also in combination with the UG1 filter; in cases with weak TB fluorescence a Zeiss 44 barrier filter, or no secondary filter at all, was used alternatively. For documentation of injections sites the extent of TB fluorescence was indicated on camera lucida drawings of adjacent Nissl stained sections. The location of TB or 5HT containing cells were manually plotted onto the standardized outlines of the appropriate brain stem level obtained from camera lucida made outlines of Nissl stained sections. Both injection sites and the positions of labeled cells was then transferred to the standardized outlines shown in the figures. Although injections were made on either side of the spinal cord, they have all been represented on the right hand side in the figures. Determination of cell sizes were made from photomicrographs with known magnification or by using a measuring ocular, cell numbers were estimated by counting cell profiles larger than 10 μm , and correcting for double counting by applying the formula of Abercrombie¹.

Results

I. Comments on the nomenclature and distribution of 5HT cells in the caudal brainstem

In the caudal brainstem spinal-projecting 5HT cells were found to be exclusively located in the ventromedial regions extending from a level just rostral to the pontomedullary junction to the caudal end of the medulla. Therefore, the areas at these levels in which labeled neurons were plotted can be conceived of as roughly triangular fields indicated in (Fig. 1a, levels I-X). Retrogradely labeled cells that were located outside this area were plotted onto the figures only if they seemed to be part of a group of cells that extended into the triangular area.

Starting at the ponto-medullary junction (levels I and II) and moving in a caudal direction gradually more 5HT cells can be seen in a medial position among the fibres of the medial lemniscus overlying the fibre-bundles of the trapezoid body. The most medially located neurons have here been considered to lie within a rostral continuation of the nucleus raphe magnus (RMR) which dorsally and laterally is surrounded by the nucleus reticularis pontis caudalis (RPC). We feel that the demarcation of the RMR as a region separate from the main nucleus raphe magnus is justified in view of the lower density of neurons and the prominence of thick fibre bundles in the former area. The 5HT cells seen at these levels are medium-sized (30-45 x 15-25 μ m) and of fusiform or multipolar appearance. At level III the 5HT-containing neurons become more numerous and begins to spread laterally towards and sometimes above or below the nucleus of the trapezoid body. These cells exhibit a differential morphology depending on their location. Thus, the cells close to the midline are mostly multipolar while the more laterally situated neurons are of fusiform type with their long axis oriented in the horizontal plane. All these ventrally located 5HT-cells (levels I-III) belong to the B3 cell group as described by Dahlström and Fuxe²⁵. At the level of the rostral part of the facial nucleus (level IV) the raphe magnus nucleus (RM), as defined here, is bordered laterally and dorsally by the nucleus reticularis gigantocellularis (RGC), the ventromedial part of which is called the pars of the nucleus reticularis gigantocellularis (RGC α)⁷⁵, while the more lateral area which laterally abuts on the facial nucleus is the nucleus reticularis paragigantocellularis lateralis (PGCL)⁷. At this level five more or less distinct groups of 5HT cells can be distinguished. Dorsomedial to the pyramids many 5HT cells are found within RM, most of which are multipolar but the lateral ones gradually acquiring more of the fusiform appearance which is very characteristic of the 5HT cells overlying the pyramids while being located within RGC. More laterally a cluster of, mostly multipolar, 5HT cells can be seen lateral to the pyramid and medial to the facial nucleus i.e. within the PGCL. Disregarding whether these 5HT cells are located in the areas we have defined as RM, RGC α or PGCL they have all been classified as B3 cells. A different type of 5HT-containing cell is seen at this level. They are located very close to the ventral surface of the brainstem, ventral to the pyramid and PGCL, and distinctly smaller (about 15x20 μ m) than most other 5HT cells seen in the CNS and sometimes exhibits a marked variation in fluorescence intensity from cell to cell. Depending on their rostrocaudal position they have been included in the B1 or B3 groups. Most of these cells are located in what Olszewski and Baxter⁵⁴ have described as the arcuate nucleus; in the following they will therefore collectively be referred to as the arcuate serotonin cell group⁴⁰. The most medially situated of the

Figure 1. (a) A series of schematic drawings of horizontal sections approximately 0.45 mm apart starting at the caudal pons (level I) and covering the distance to the pyramidal decussation (level X). The hatched lines delineate the various nuclear regions studied. The triangular outlines define the area within which labeled cells were recorded.

In (b) the distribution of 5HT cells seen in one animal is given at levels I-X. Each dot represents one 5HT cell in a 15 μ m section at the corresponding level. Light stippling indicates areas with high density of CA-fibers and terminals.

Abbreviations: Am, nucleus ambiguus; DP, pyramidal decussation; IO, inferior olive; LR, lateral reticular nucleus; MeV, mesencephalic trigeminal nucleus; MoV, trigeminal motornucleus; NIH, nucleus interfascicularis hypoglossi; PGCL, nucleus paragigantocellularis lateralis; PrV, principal trigeminal nucleus; RGC, nucleus reticularis gigantocellularis; RGC α , nucleus reticularis gigantocellularis, pars α ; RM, nucleus raphe magnus; RMr, rostral extension of nucleus raphe magnus; RO, nucleus reticularis pontis caudalis; RV, nucleus reticularis ventralis; SCP, superior cerebellar peduncle; SO, superior olive; SpV, spinal trigeminal nucleus; ST, subtrigeminal nucleus; TB, nucleus of the trapezoid body; VII, facial nucleus and nerve root.

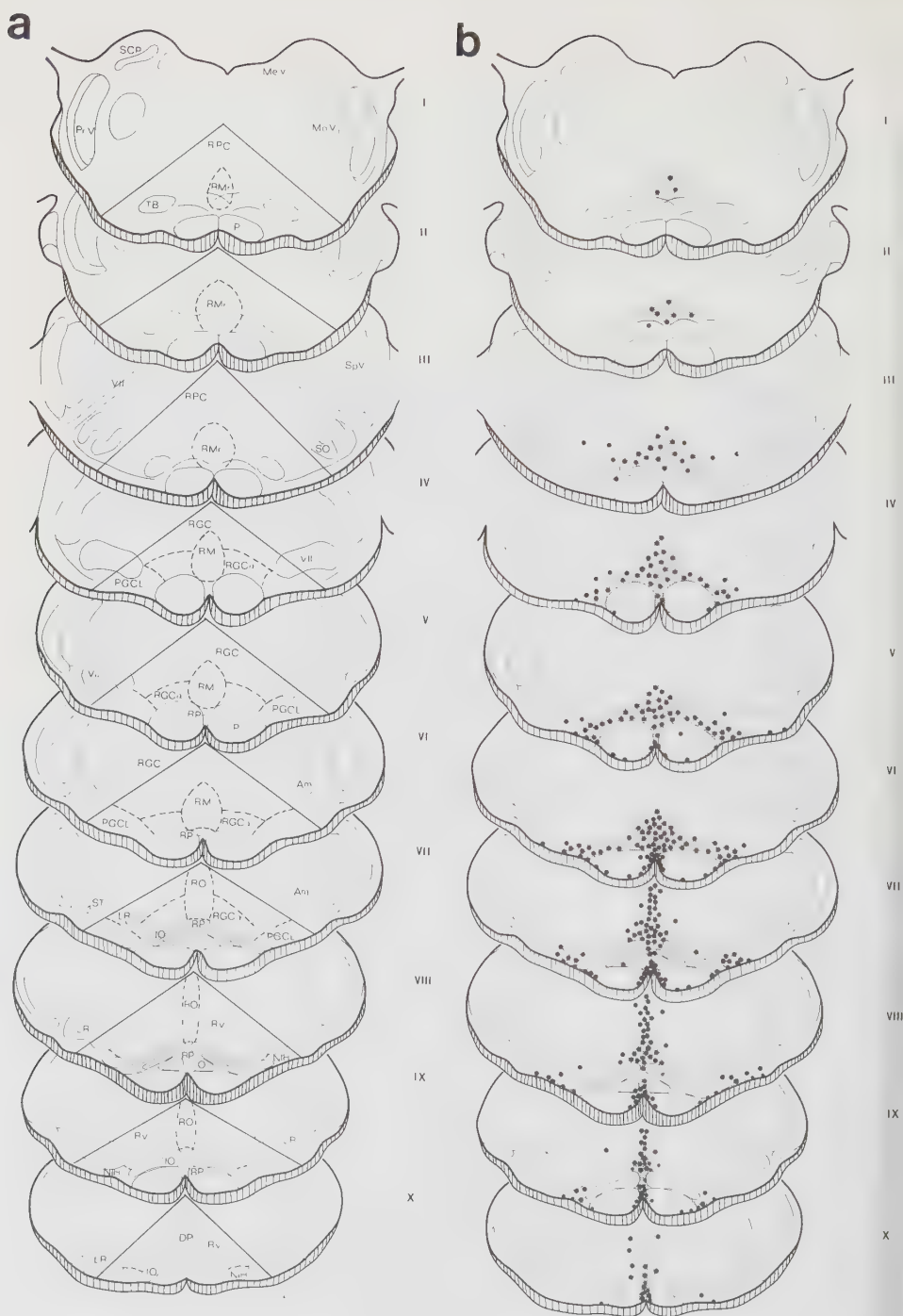


Fig. 1

arcuate cells borders on neurons within the nucleus raphe pallidus (RP), which is a cluster of cells located between the pyramids and more caudally extending dorsally between the inferior olives. The 5HT cells in the RP (group B1 of Dahlström and Fuxe) are round and usually somewhat smaller than the cells in RM but clearly of greater diameter than the cells in the arcuate region. At level V, which corresponds to the caudal part of the facial nucleus, the number of 5HT cells in the RGC decrease so that the multipolar 5HT cells tend to form separate medial (within RM) and lateral (within RGCL) cell clusters.

At level VI, the medially located 5HT cells are contributed to by all three medullary raphe nuclei; while the most ventrally situated cells belong to RP, the more dorsally distributed cellbodies are located within RM, though some of these cells has the appearance of belonging to the rostral part of the nucleus raphe obscurus.

Proceeding caudally we find that at rostral levels of the inferior olive (level VII) the medially located 5HT-cells situated dorsal to the dorsal most level of the inferior olive (level VII) belongs to the nucleus raphe obscurus (RO) within which most cells are fusiform and bilaterally distributed in two longitudinal rows close to the midline and dorsally extending into the fibre bundles of the medial longitudinal fasciculus. The 5HT-cells in this nucleus are typically fusiform with their long axis oriented in the vertical plane. At this and at more caudal levels the area just lateral and dorsal to the lateral tip of the inferior olive has been named the nucleus interfascicularis hypoglossi (NIH)^{23,54}. In this area a cluster of fusiform or multipolar 5HT cells are seen constituting the lateral part of the B1 group. Here, as well as at more rostral levels, these cells gradually merge with the arcuate neurons sometimes making it difficult to categorize cells as belonging either to PGCL/NIH or to the arcuate group. We have assigned to the arcuate cell group only those cells which have a diameter of less than 20 μm and which are situated immediately adjacent (within 25 μm) to the ventral surface. The arrangement of 5HT cells remains essentially unchanged through levels VIII and IX where the spinal projecting, mostly non-5HT cells dorsal to the pyramids and lateral to the RO forms the ventral part of the nucleus reticularis ventralis (RV). At the level of the pyramidal decussation (level X) the 5HT cells rapidly decrease in number, most of them being round and located in RP, close to the midline ventromedial to the decussating pyramidal fibres. A smaller number of multipolar neurons are located in RV just lateral to the decussation. Usually only a few 5HT cells belonging to the NIH or arcuate nucleus are found at this level. All 5HT cells at this the most caudal level studied belong to the B1 group. These general findings on the distribution and appearance of 5HT-containing cells in these regions are in good agreement with other studies using similar or different techniques^{15,25,61,62}.

II. Distribution of catecholamine fibres

In the animals that were not pretreated with a MAO-inhibitor (and consequently showed very little 5HT-fluorescence) the location of catecholaminergic fibres within the ventromedial caudal brainstem were mapped. The areas with particularly rich catecholaminergic innervation are indicated in Fig 1b as stippled areas. Already at a glance it is clear that the dense distribution of catecholamine-fibres overlaps to a high degree with the location of 5HT-containing cell bodies. It must, however, be noted that, with the exception of the pyramids and the trapezoid body, the areas that are not stippled in the figure were

not devoid of catecholamine fibres but exhibited a fairly even background plexus, albeit with a much lower density. Furthermore, parts of the inferior olive were densely supplied with CA fibres, but since this structure is not known to have any spinal projections this innervation has not been indicated in the figure.

III. Injection sites

In most cases studied the tracer was deposited at lower cervical or thoraco-lumbar levels. Since it has been shown that some 5-HT neurons give rise to supraependymal fibre plexa (Richards et al. 1981) all injection which showed signs of leakage of the tracer substance into the subarachnoid space were discarded from the material. After having excluded such injections tissues from about 60 animals remained. The majority of these successful injections had been placed at low cervical or at thoraco-lumbar levels.

By comparing the visual appearance of the injection site at 365 and 435 nm excitation the extent of a peripheral and a central zone of TB deposition could be determined. The central zone was defined as the central part of the injection site where the normal cytoarchitecture was completely obliterated and replaced by intense TB fluorescence that was easily discernable even at 435 nm excitation. The peripheral zone constitutes the area that did not exhibit any TB fluorescence when activated at 435 nm but which showed intense and homogenous tracer fluorescence at 365 nm excitation. The peripheral zone gradually merges with the normal tissue where tracer fluorescence is seen only intracellularly and usually only at 365 nm excitation. Fig. 2 illustrates the appearance of an injection site as visualized at either excitation wavelength. In the schematic figures the central zone is black and the peripheral zone is indicated by the lighter screen. Often the injection site had an irregular shape and in order to document its total extent the pictures obtained from several closely spaced sections has been superimposed in the figures. In most cases the injections were confined to the segment indicated, if they were judged to extend into adjacent segments this has been indicated in the text. Identification of the segmental levels of the injections was in most cases readily made on the basis of the configuration of the grey and white matter and the localization of big motoneurons. At thoracic levels, however, this method was sometimes not sufficient for positive identification of the segment, the level indicated for the thoracic injection sites are therefore only approximate (* one to two segments).

Appearance of 5HT and TB in the retrogradely labeled cells

The identification of 5HT-containing cells were based on fluorescence characteristics; 5HT cells exhibits a diffuse, cytoplasmic yellow-brown fluorescence where the yellow component characteristically shows a fairly rapid fading upon exposure to either 365 or 435 nm light, the fluorescence colour and the fading characteristics clearly separate the 5HT cells from the more green-yellow fluorescence of catecholamine-containing neurons, which does not show appreciable fading upon exposure to 365 or 435 nm. Non-5HT cells that are retrogradely labeled by the fluorescent tracer TB show up as cell bodies filled with fluorescent granules exhibiting the blue or green fluorescence (depending on the secondary filter used) typical for TB when excited at 365 nm. At 435 nm excitation the intracellular TB-contain-

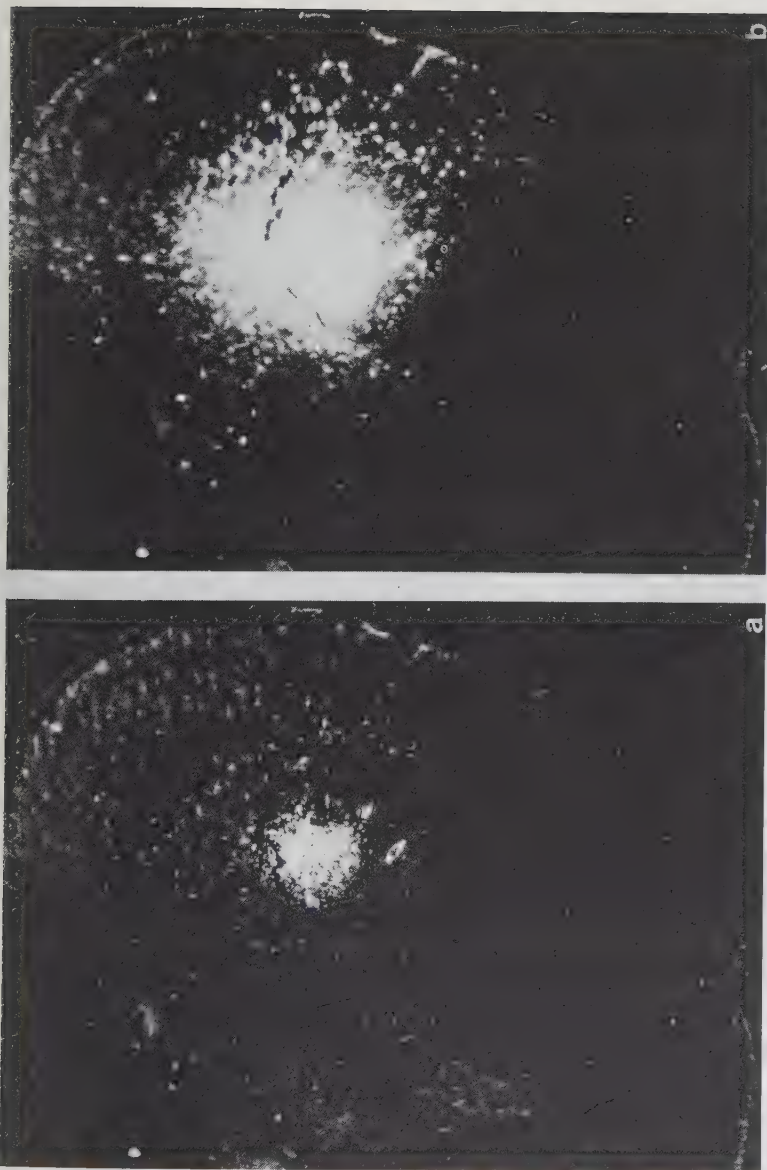


Figure 2. Photomicrograph illustrating a small injection site at an upper cervical segment just lateral to the reticular nucleus of the spinal cord. Half of the spinal cord is seen, the dorsal horn is to the upper right. (a) At 435 nm excitation only the central zone of the injection site is seen. Some fluorescent monoaminergic fibers can be seen in the dorsal and ventral horn. In (b) the same field of view is seen at 365 nm excitation which visualizes also the peripheral zone of the injection.

ing granules does not show appreciable fluorescence and thus the cells disappear and reappear from the microscopic image as the excitation light is shifted from 365nm to 435 nm and back again.

In the case of retrogradely labeled 5HT cells, these neurons present the typical 5HT fluorescence when excited at 435 nm. As the excitation is changed to 365 nm the fluorescence will, if the retrograde labeling is heavy, typically exhibit an increased intensity and become more granular in appearance (Fig. 3). In cases of weaker labeling the increase in fluorescence intensity and granularity may not be appreciable. The labeling can then only be verified by the colour of the fluorescence emission at 365 nm excitation: the yellow-brown fluorescence of the weakly labeled 5HT cells will then assume a bluish colour when the excitation is switched to 365 nm.

V. Distribution of retrogradely labeled cells after spinal injections

Large midcervical injections

These injections attempted to completely fill one half of the spinal cord at the level of the cervical enlargement. By delivering the tracer through several penetrations uptake could possibly occur both through terminals in the grey matter and via severed axons in the white matter. These injections may thus provide an overview of the spinal cord projecting neurons associated with the brain stem 5HT cell groups projecting to midcervical or lower spinal levels.

Retrogradely labeled cells were distributed in the mesencephalon (close to the dorsal raphe nucleus and to a lesser extent also in association with other mesencephalic 5HT cell groups), in the caudal pons (in the area of the rostral extension of RM) and in the medulla, where all three raphe nuclei and adjacent regions were heavily labeled.

In Figs. 4a and 13 the distribution of retrogradely labeled cells in the regions examined is plotted from animal no. 08-12 which received such a large midcervical injection. Quite characteristically, most of the contralaterally labeled non-5HT cells, exhibited a lower fluorescence intensity than the ipsilaterally labeled ones. This phenomenon of weaker labeling contralaterally was not only observed in this animal but was noted in most experiments. The shape of the neurons did not differentiate between 5HT and non-5HT cells but could generally be accounted for by their location. Thus, the labeled cells (both serotonergic and non-serotonergic) which are lying close to the dorsal surface of the pyramids were mostly horizontally oriented, fusiform and medium-sized to large (30 μ m or more), while more medially or dorsally located cells were typically multipolar. The cells close to the ventral surface of the brain were consistently small and round to fusiform regardless of the nature of their transmitter content.

In the mesencephalon the number of TB labeled cells in association with 5HT cell groups were low compared to the total number of 5HT cells present at these levels and also when compared to the number of retrogradely labeled cells at medullary levels. It is noteworthy, though, that about half of these labeled cells were 5HT-containing (see Section VII, below).

In the pons the region of the raphe pontis nucleus was in all cases devoid of retrogradely labeled cells, while more caudally TB labeled cells in this animal were first seen within, or close to, the RMr and the adjacent RPC. Single 5HT cells could be seen to be retrogradely labeled, especially ipsilaterally, already at level I and increasing in number down through level III resulting in retrograde labeling of almost half of the population of 5HT neurons through these levels.

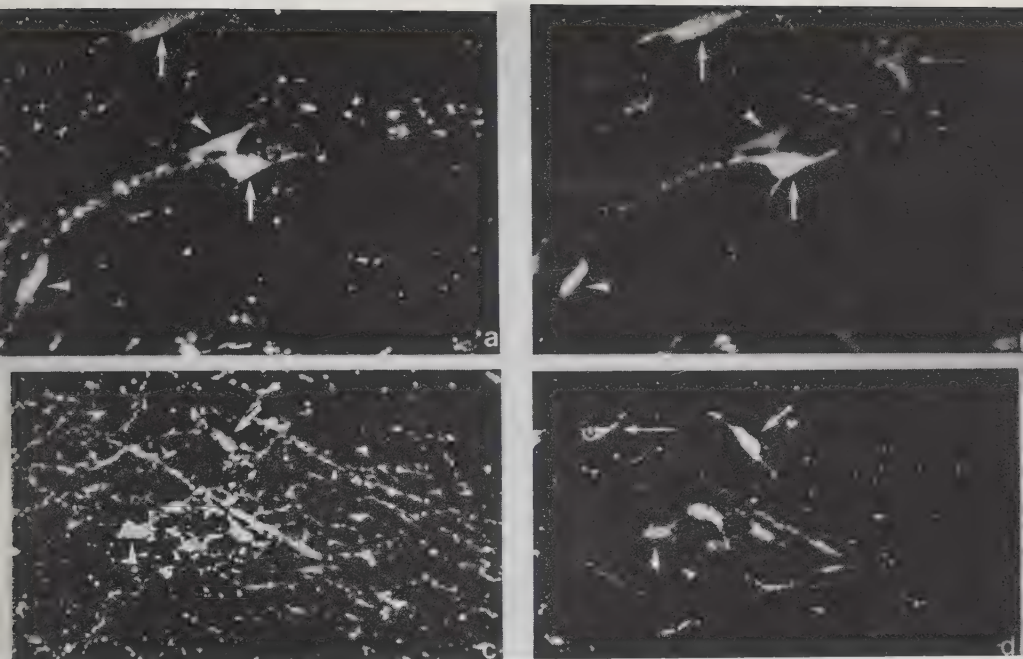


Figure 3. Appearance of labeled and unlabeled cells in the region of RGC after an injection into the dorsal horn at T_5 . In (a) excitation at 435 nm visualizes only 5HT-containing cells and monoaminergic fibers. Excitation at 365 nm (b) visualizes preferentially TB; 5HT-containing cells which have accumulated the tracer show an increased fluorescence intensity and granularity (thick arrows). 5HT cells that have not taken up TB instead show a decrease in intensity (arrowheads). The slender arrow points to a retrogradely labeled non-serotonergic cell, this neuron can not be seen at 435 nm excitation. X320. The two lower micrographs also illustrate the dense catecholaminergic innervation of the area. (c) and (d) show the fluorescence at excitation at 435 and 365 nm, respectively. Arrows as in (a) and (b) X 142.

From the figure it is also evident that there is an, equally abundant and partly bilateral non-5HT projection from the same area and also from cells lying dorsal and lateral to the 5HT cells. At the level of the facial nucleus (Fig. 4a, level IV, V) the retrogradely labeled 5HT cells in the RM, RGC α and PGCL constituted about 30% of the total 5HT population in these nuclei on one side and were seen to be strictly ipsilaterally located. A larger number of TB labeled non-5HT cells were preferentially located in the ipsilateral RGC, dorsal to the 5HT cells, but labeled non-5HT cells were also often seen intermingled with labeled and non-labeled 5HT cells more ventrally in the RM, RGC and PGCL and, to a lesser extent, also in similar positions contralaterally.

In the medulla oblongata, neurons in the PGCL were labeled bilaterally at levels IV-VII, but the 5HT cells were labeled only ipsilaterally where they constituted about half of the labeled cells. In the RP at these levels only perhaps 30% of the 5HT neurons were labeled and tended to be outnumbered by the retrogradely labeled non-5HT cells in this nucleus. In the RM at level VI about half of the 5HT cells in the ipsilateral nucleus were retrogradely labeled as were the case for an occasional serotonergic neuron contralaterally. Only few 5HT cells were labeled in RGC α , while many labeled non-5HT cells were seen in this area and, more dorsally, in RGC. In the ipsilateral PGCL about half of the 5HT-containing neurons were labeled together with a similar number of non-5HT cells. A similar number of non-5HT cells were seen also in the contralateral nucleus. In the arcuate nucleus most of the ipsilateral 5HT neurons were labeled; in RP still only a minority serotonergic neurons contained the tracer but a slightly higher number of labeled non-5HT cells were seen.

At level VII around half of the 5HT cells in the ipsilateral RO were retrogradely labeled. In this area about an equal number of retrogradely labeled non-5HT neurons were seen, these were, however, bilaterally distributed and were usually slightly more laterally situated. In the ipsilateral RGC α and RGC many labeled non-5HT cells were located dorsal to the inferior olive, contralaterally labeled neurons were seen in a corresponding position: they were, however, fewer and did exhibit a lower fluorescence intensity. In the ipsilateral PGCL most of the few 5HT neurons contained TB as did a higher number of non-5HT cells located dorsolaterally to the 5HT cells. In the arcuate nucleus the labeling was very similar to that seen at the adjacent levels. In RP still only few cells were labeled but now with some dominance of labeled 5HT cells to non-5HT cells.

At levels VIII and IX the relative proportion of TB labeling in RO (and adjacent RV) and RP increased to reach about 30-40% of total the population of 5HT cells in these nuclei. They were distributed on both sides of the midline with an ipsilateral preference, in RP intermingled with, but in RO most often located medial to, a lower number of retrogradely labeled non-5HT cells (Fig. 14). Labeling in PGCL was almost exclusively ipsilateral as the number of TB-containing non-serotonergic cells decreased caudally. In the arcuate nucleus most ipsilateral 5HT cells were labeled, and occasional labeled non-5HT cells were also seen. The labeled non-5HT cells in the RV dorsal to the inferior olive were on both sides seen to bridge the gap between the medial and lateral 5HT cells. As the corticospinal fibres began to decussate (level X) the number of labeled cells decreased rapidly, while the fraction of labeled 5HT cells increased to amount to about 50% of the total 5HT cell population at this level. Information on the

total and relative number of labeled 5HT and non-5HT cells that were seen after this and similar injections at L6 and S4 are given in table I and in Section VI, below.

Injections into subregions of the spinal cord at cervical levels

After injections into restricted regions of the cervical cord generally fewer neurons were found to be retrogradely labeled in the brainstem areas investigated and the distribution of labeled cells varied markedly depending on the region injected. Thus, injections confined to dorsal parts of the cord produced a pattern of labeling at rostral pontomedullary levels similar to that seen after complete hemichord injections, while only sparse labeling was seen at lower medullary levels. After injections to ventral cord regions this pattern was essentially reversed with the majority of labeled cells seen at caudal levels. While injections into the lateral funiculus could produce labeling in all brainstem regions studied it is notable that injections that covered medial parts of the cord consistently failed to label any neurons in the arcuate nucleus. With respect to injections into the lateral funiculus there was a differential distribution of labeled cells in the brainstem depending on which part of the lateral funiculus was injected; While injections into the dorsal part of the lateral funiculus labeled cells in RM and RMr but only few cells in RP, RO and the arcuate nucleus progressively more cells were seen in these areas when the injections covered more ventral parts of the funiculus. When the deposition of tracer was restricted to the grey matter a striking feature was the decrease in number of labeled cells and, seemingly especially pronounced in the 5HT cells, the reduced intensity of the TB-labeling.

In rat 15-02 (Fig. 4b) a small injection covered the most dorsal part of the lateral funiculus and it also encroached upon the lateral tip of the dorsal horn. Compared to the hemichord injection in rat 08-12 (above), the most obvious difference, besides the marked decrease in the overall number of labeled cells, was the virtual absence of labeled cells, especially 5HT-containing ones, in RP, RO and the arcuate nuclei. Only few cells were seen in RGC and the contralateral labeling was reduced throughout.

Fig. 5a shows the retrograde labeling in rat 06-25 after an intermediate sized injection which involved a larger part of the lateral funiculus at C7. Again the relative absence of contralateral labeling, at most levels, was striking, but compared to rat 15-02 there were some labelled cells in RP and RO and the number of TB-labeled neurons in the arcuate nucleus was quite significant (Fig. 15).

Rat 04-30 received a small superficial injection which covered the superficial part of the lateral funiculus and it extended further ventrally than the two latter ones. In this animal (Fig. 5b) the ipsilateral dominance of labeled cells was striking but the only region completely devoid of TB-labeled cells was the nucleus RO and medial parts of RV.

Fig. 6a (rat 09-12), illustrates a large injection that was complementary to the ones in the lateral funiculus by filling the more medial parts of the cervical segment. While producing a general pattern of labeling quite similar to that seen after the large hemichord injection in rat 08-12 there was a fairly even decrease in number of labeled 5HT and non-5HT cells in most areas but with a more marked reduction of labeling in the arcuate nucleus.

Figures 4-12. Representations of some injection sites and corresponding distribution of retrograde labeling at caudal pontine and medullary levels. At each level is plotted the number of cells seen in one corresponding 15 μm section. Each filled star represents one retrogradely labeled 5HT cell, unfilled stars represent labeled non-serotonergic cells. Large unfilled stars represent five labeled non-serotonergic cells. (In figures 4a and 10b the retrograde labeling is slightly underrepresented, see table I for absolute numbers of labeled cells in these cases). On top of each series of drawings is given the code number of the rat.

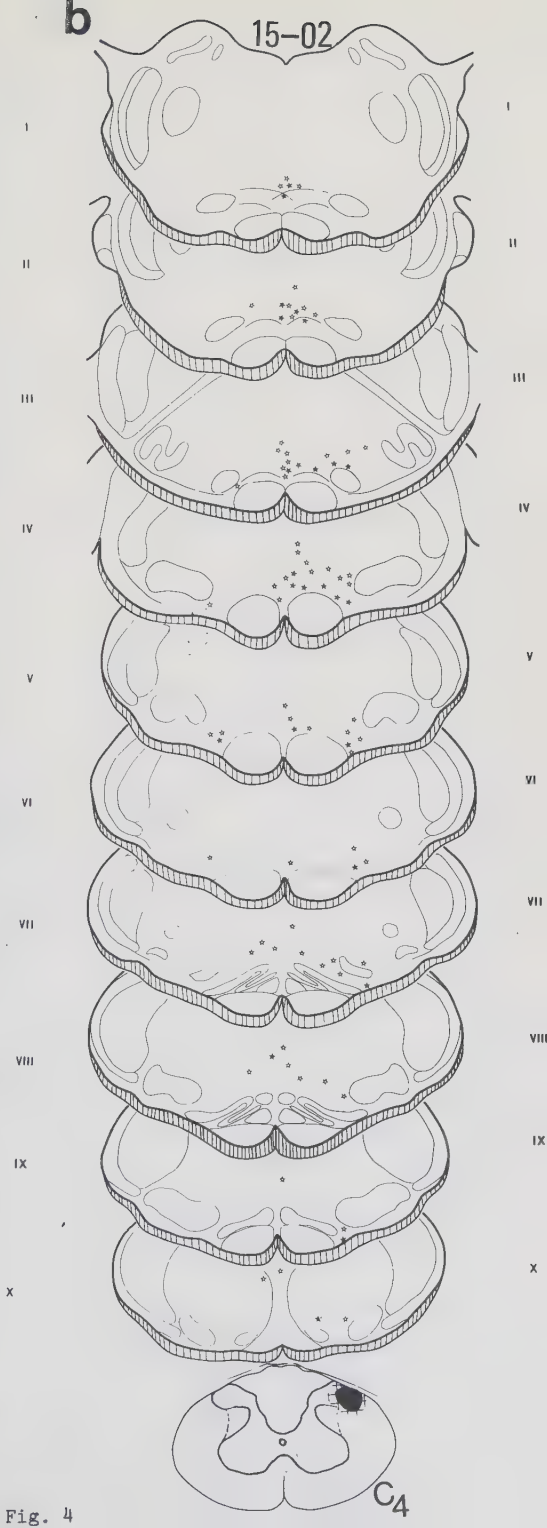
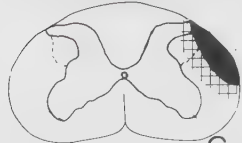
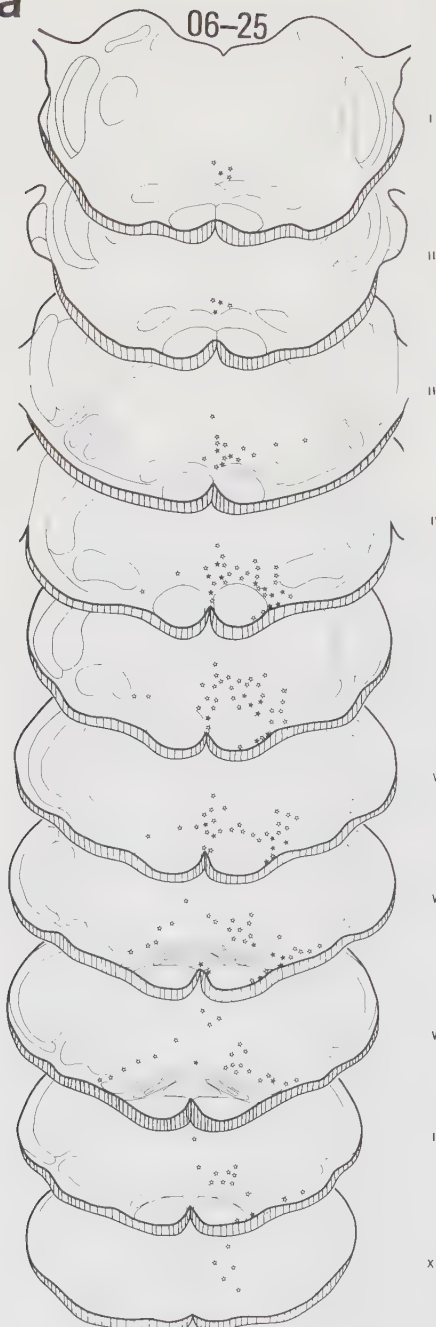


Fig. 4

a

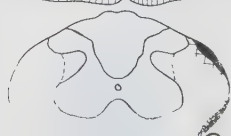
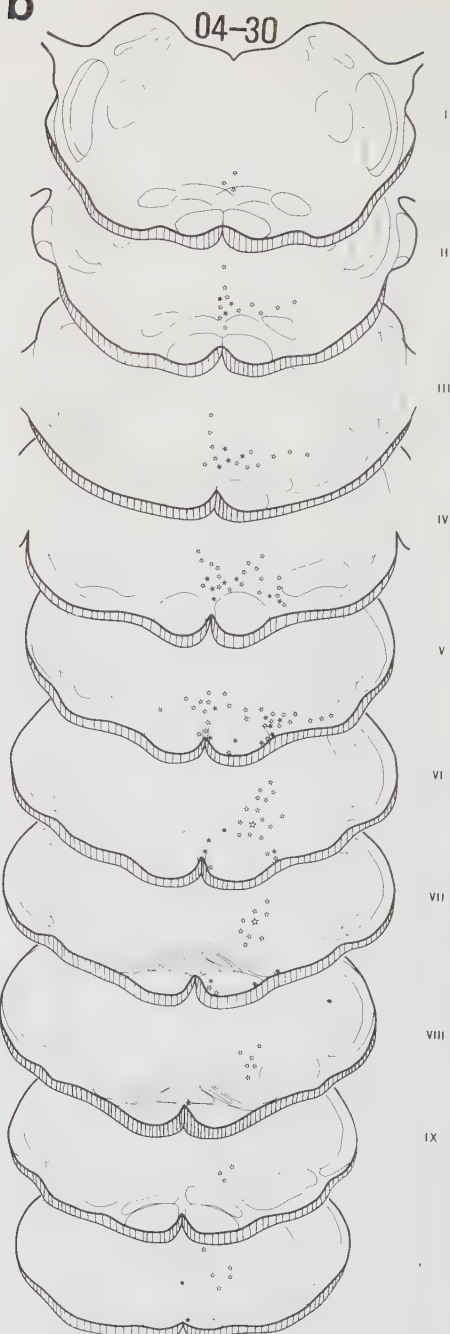
06-25



C7

b

04-30



C4

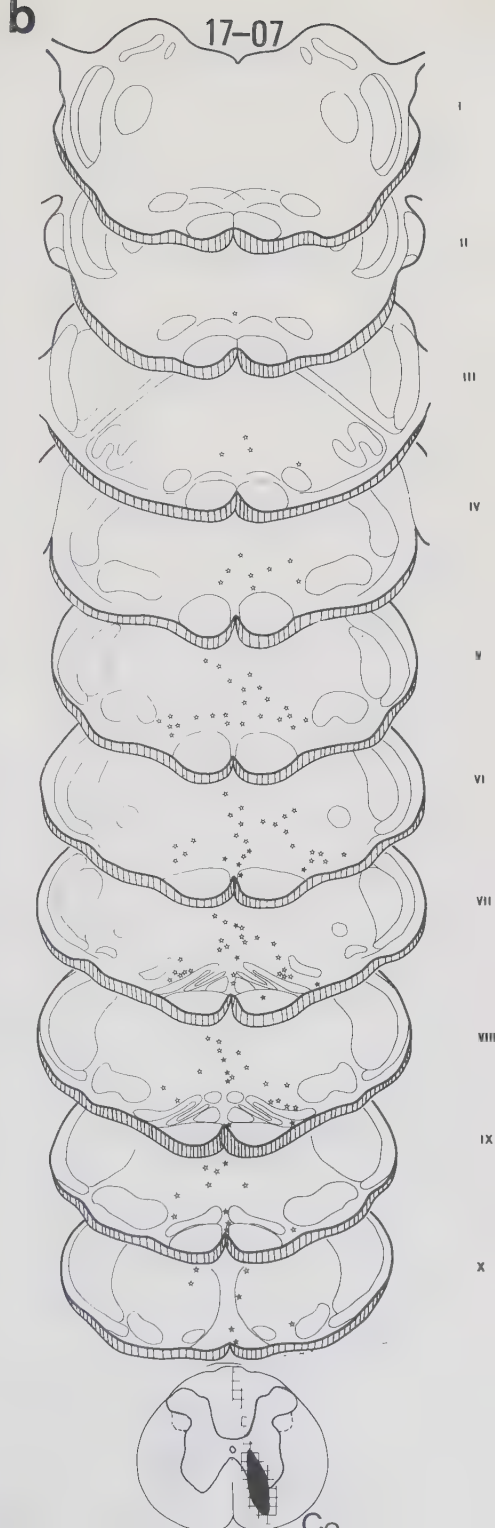
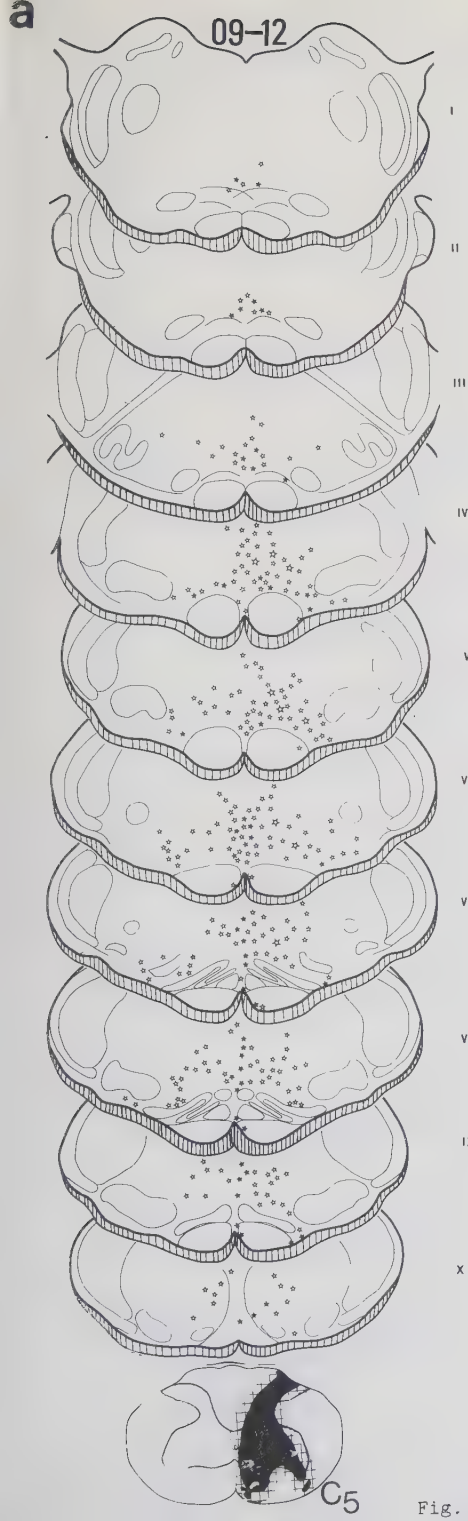
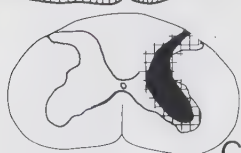
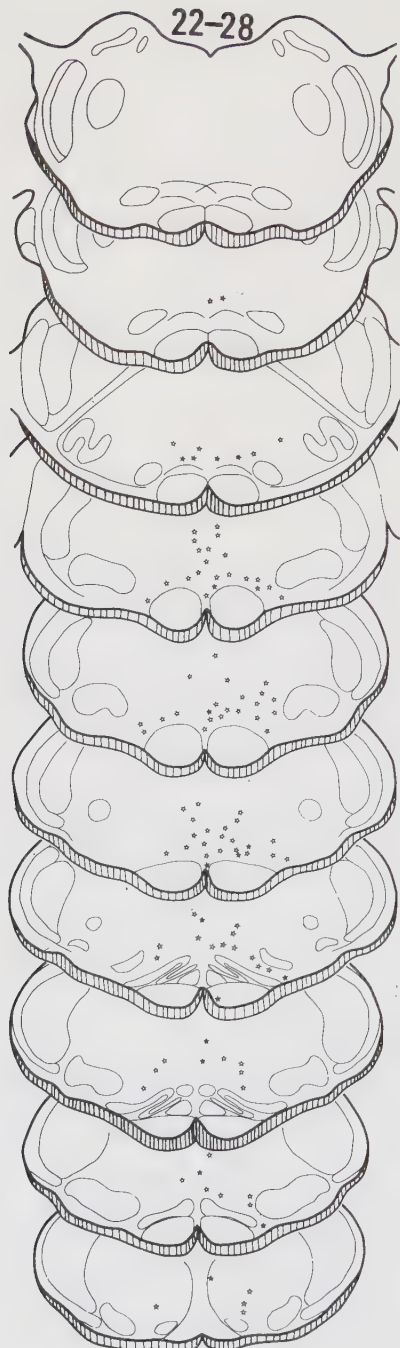


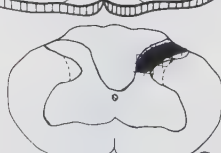
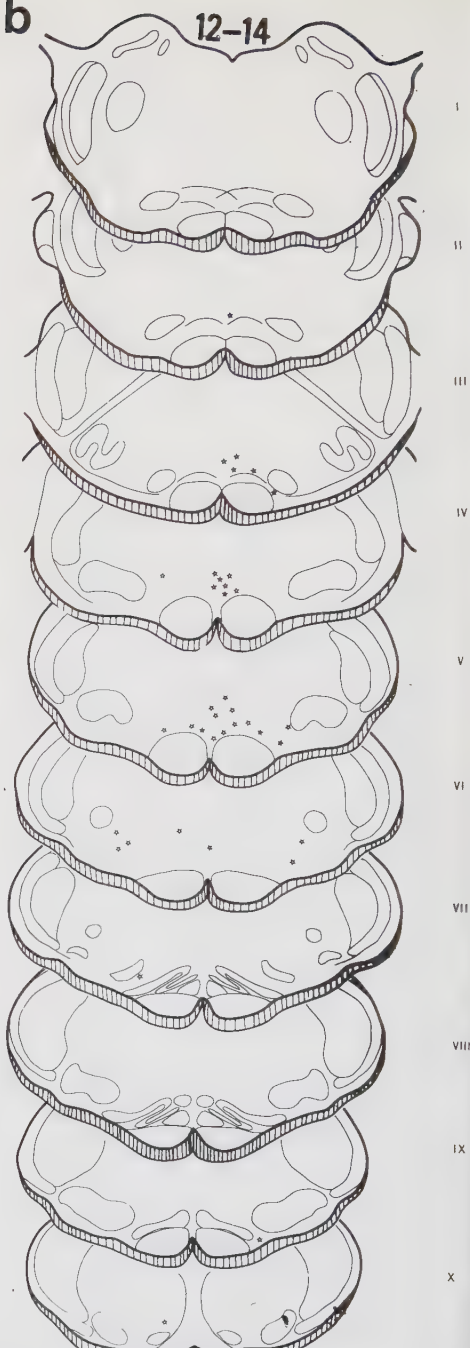
Fig. 6

a

22-28

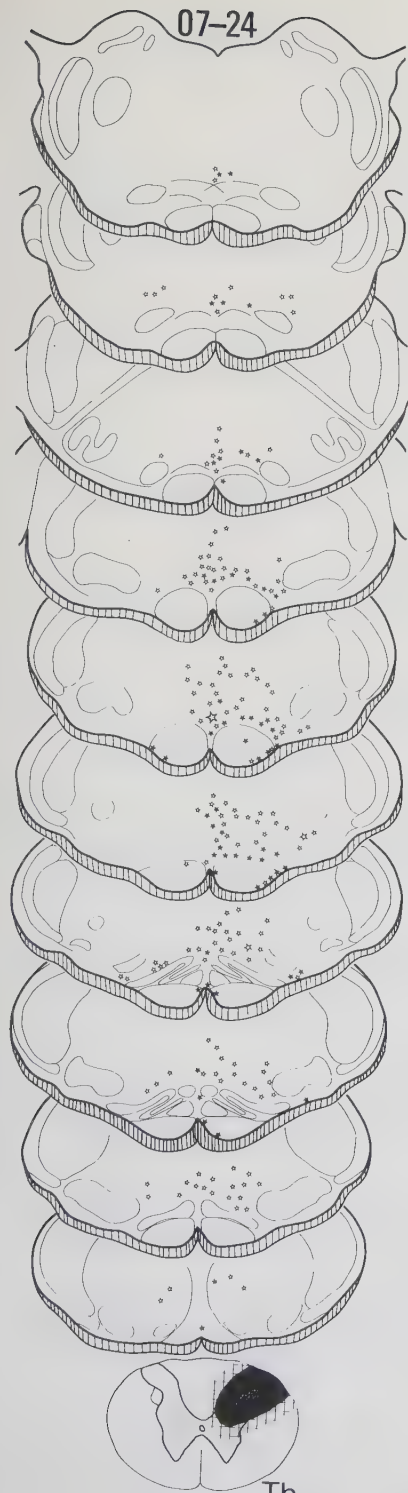
**b**

12-14



a

07-24

**b**

03-21

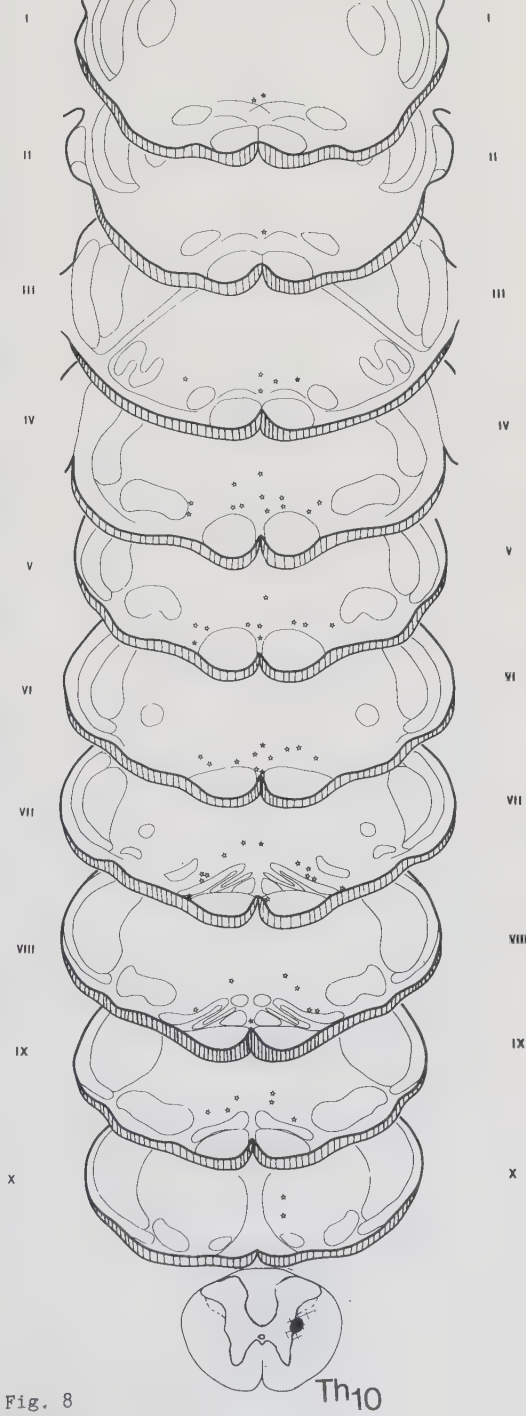
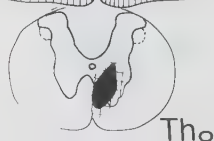
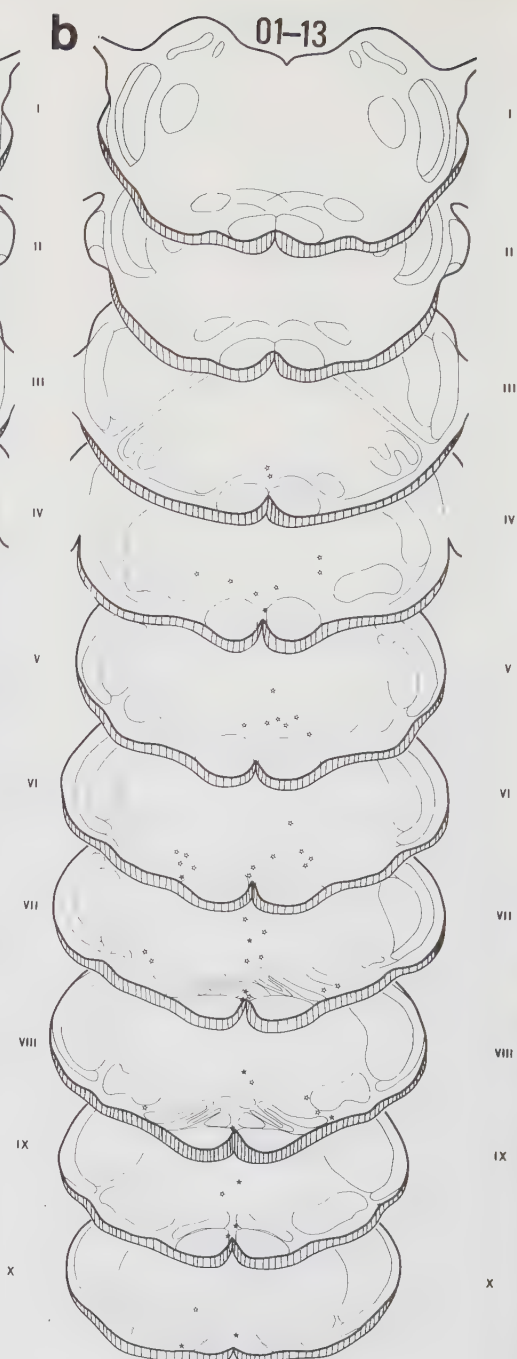
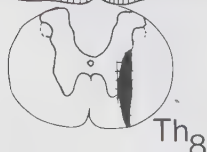
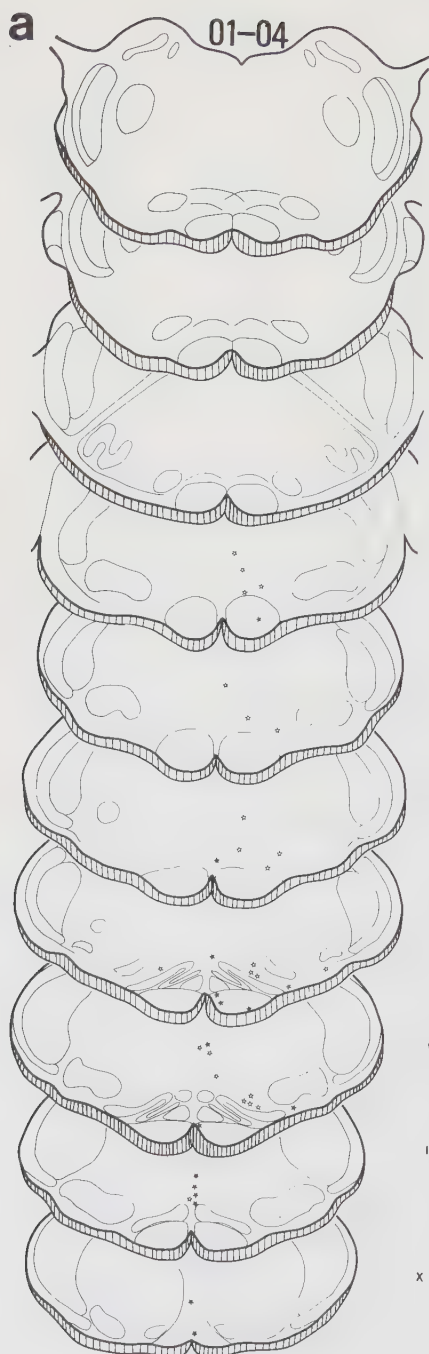


Fig. 8



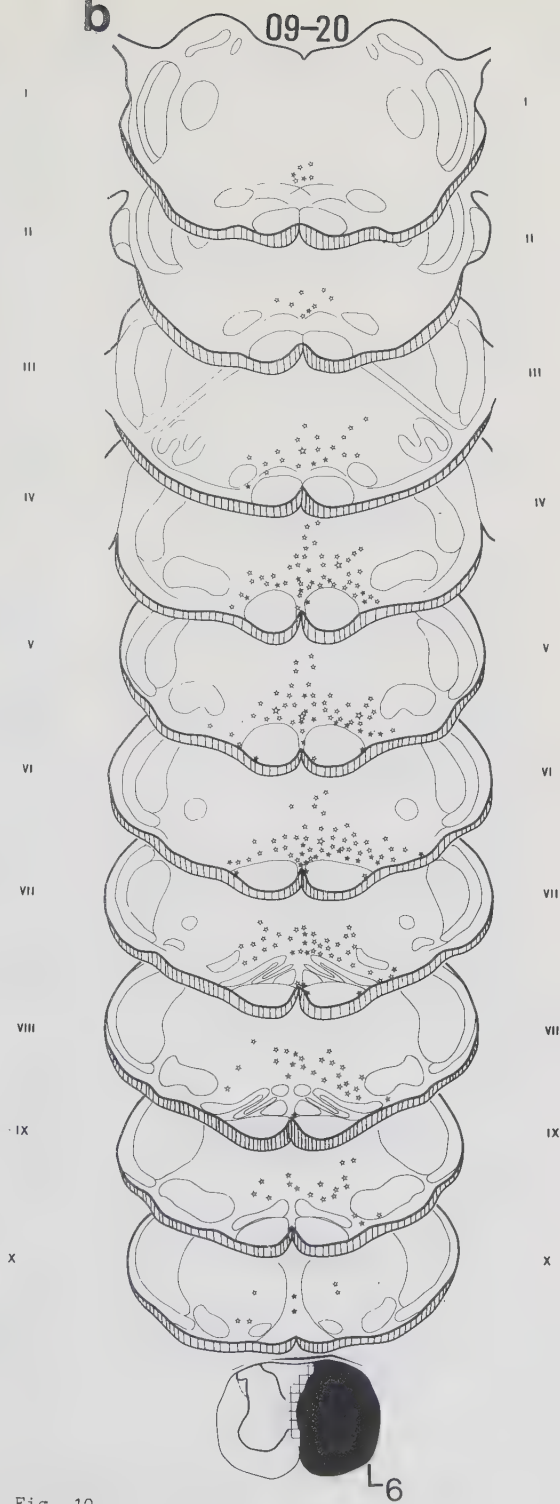
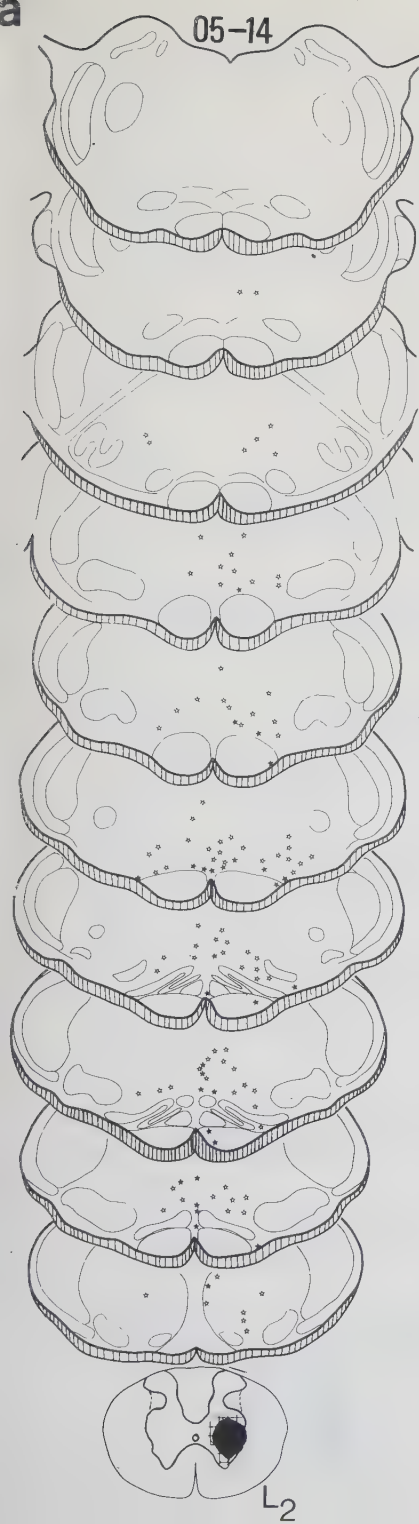


Fig. 10

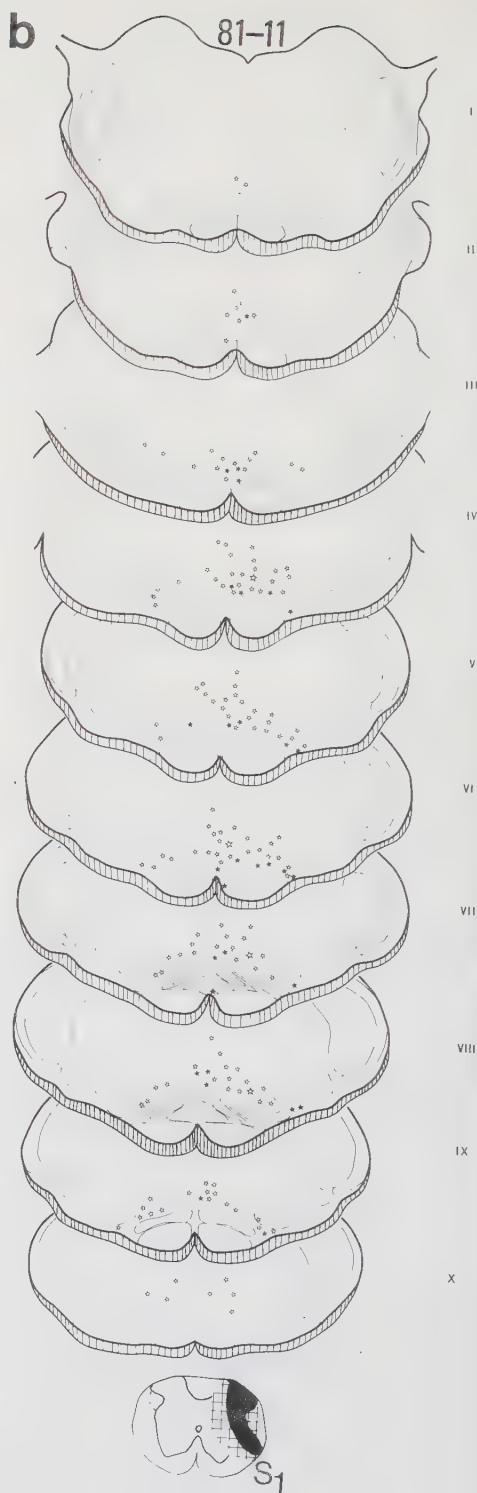
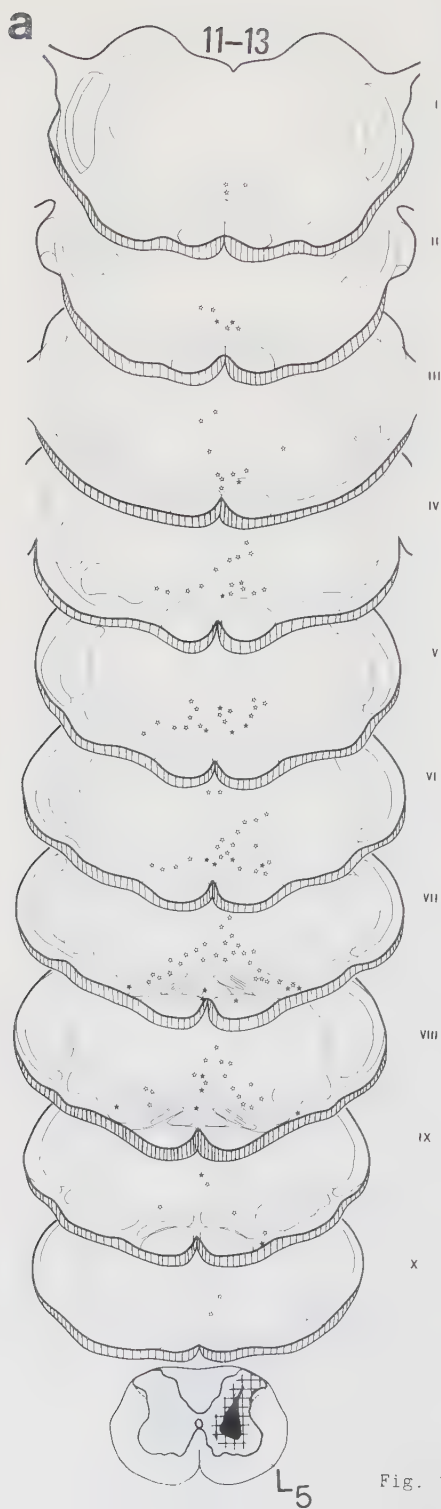


Fig. 11

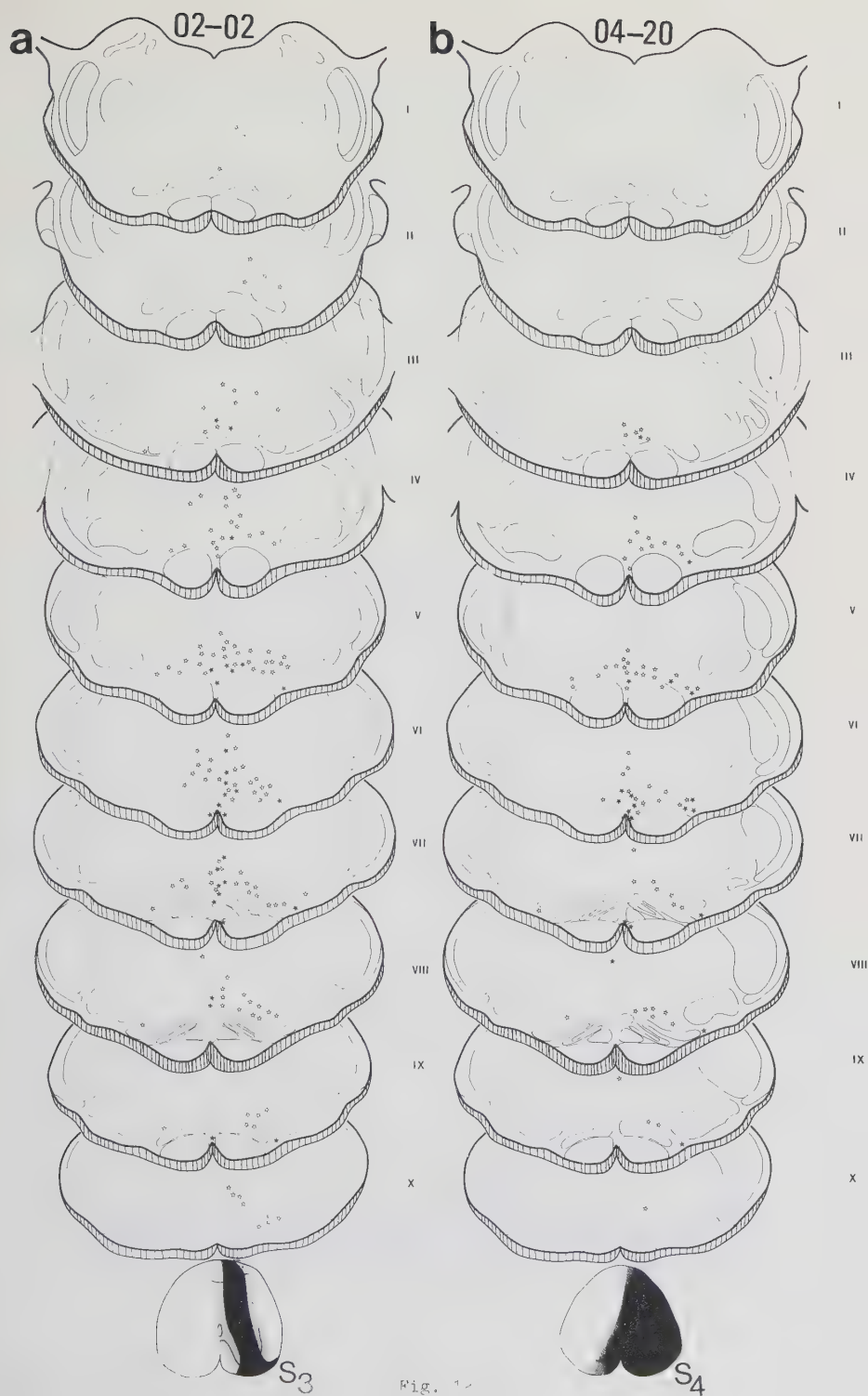


Fig. 1

With injections limited to the ventral part of the cervical cord labeled 5HT cells were found only at levels caudal to the facial nucleus. Labeled non-5HT cells, on the other hand, could be seen also at more rostral levels. The labeled 5HT cells seen after these injections were almost exclusively located medially in RP and RO while virtually no such cells were found laterally. In rat 17-07 the injections at the third cervical segment covered the medial part of the ventral horn and the adjacent area of the ventral funiculus (Fig. 6b). In this animal only very few retrogradely labeled cells were found at the levels of RMr. At levels IV-VI a moderate number of TB-labeled non-5HT cells were seen in RGC, RM, RGC α and PGCL, especially ipsilaterally. Of these labeled neurons almost all were situated dorsal to the location of 5HT-containing cells. In this experiment it was not until level VI that a small number of 5HT cells were retrogradely labeled. More caudally a moderate number of TB-labeled cells were found throughout the length of RP and RO, some of which were 5HT-containing. Occasionally, such cells were also seen in the caudal PGCL. At these caudal levels (VII-X) the majority of labeled cells were non-5HT-containing and showed a marked tendency for being located dorsal to the 5HT cells. A notable exception was the bilaterally occurring small cell clusters found just dorsal to the dorsal nucleus of the inferior olive.

Rat 22-28 (Fig. 7a) received an intermediate-sized injection, essentially restricted to the grey matter. In this case labeled neurons were found in all areas that were labeled in rat 08-12 with the exception of the most rostral part of RMr and RPC and, more notably, the arcuate nucleus. Fig. 7b illustrates the labeling seen after a small injection into the dorsal horn (rat 12-14). In this animal no labeling was found in nucleus arcuatus, RP, RO or RV and only few cells seen in RM, RPC and NIH.

Injections at thoracic and upper lumbar levels

In comparison with cervical injections the thoraco-lumbar injections revealed, first, a more widespread distribution of 5HT-axons emanating from the caudal medullary levels, and, secondly, the appearance of labeled arcuate cells after injections apparently confined to the grey matter. With tracer injections confined to ventral regions of the cord at thoracic and upper lumbar levels a prominent feature was the virtual absence of labeled cells at the most rostral medullary levels while a low number of labeled cells were found in RM, RGC α , RGC, PGCL, NIH and RV and also in RO and RP. The majority of these labeled neurons were serotonergic. In the mesencephalon individual TB-labeled non-5HT cells could sometimes be seen in close proximity to the dorsal raphe nucleus but no labeled 5HT cells were ever seen in the mesencephalon after injections below cervical levels.

Fig. 8a illustrates the findings in rat 07-24 which received an intermediate sized injection at T₆ which covered the dorsal part of the lateral funiculus and the grey matter except the ventral horn. At levels I-III about 10 labeled cells were seen per section, most of these being found ipsilaterally. Of the ipsilaterally labeled cells 30-40% were 5HT-containing. At the level of the facial nucleus there was a marked increase in the number of labeled cells and with the exception of RM in which labeled cells were found on both sides (Fig. 17) of the midline, there was now a more strict ipsilateral distribution. In RM about 25% of the labeled cells were serotonergic, and they tended to be located ipsilaterally, intermingled with labeled non-5HT cells. In RGC α there was about as many labeled non-5HT as 5HT-containing cells, most of the latter occupying a slightly more

dorsal position. In PGCL there was also a similar number of both cell types while in the arcuate region the majority of labeled cells were serotonin-containing, and occasional such cells were also found contralaterally. At level VI a few labeled 5HT cells were found bilaterally in a dorsal position close to the midline, seemingly belonging to the rostral part of RO. Here, and also at level V, a number of TB labeled cells were found dorsal to RGC α on the ipsilateral side. In PGCL about an equal number of labeled 5HT and non-5HT cells were found and only at the most caudal part of this nucleus were a few of the latter found contralaterally. In RP a low number of labeled 5HT cells were seen at all levels. While this nucleus did not contain any labeled non-5HT cells such neurons were seen in RO together with a small number of labeled 5HT cells. Dorsal to the inferior olive labeled non-serotonergic cells were seen on both sides but predominantly ipsilaterally. No cells were found in NIH but a few labeled 5HT cells were observed in the arcuate nucleus at midolivary levels.

Rats which received injections involving a larger part of the lateral funiculus presented a very similar pattern of labeling although a higher number of cells, both serotonergic and non-serotonergic were seen. A few of the labeled 5HT cells were situated in the contralateral RM.

The present material does not contain any injection that was confined to the IML but the injection site illustrated in Fig. 8b (rat 03-21) represents as good an approximation of such a localized injection as could be obtained by the technique used. In this experiment heavy tracer deposition was found in the IML and in the immediately dorsolateral white matter extending about 0.3 mm along the longitudinal axis at Th₈. In this case retrogradely labelled cells were seen throughout the pontomedullary levels. The TB-labeled neurons, which were almost equally distributed on the two sides, were generally weakly labeled. With the exception of NIH all nuclear areas within the region examined contained labeled cells but relatively fewer dorsally. Of the retrogradely labeled cells seen within RM only very few, if any, were serotonergic. The positively identified 5HT cells were seen in RMr, RPC, PGCL, RP, RO and arcuate nucleus (though only a total of three such cells could be seen in the latter nucleus).

In rat 01-04 (Fig. 9a) the injection covered the most lateral part of the ventral horn and extended out in the white matter at the border zone between the ventral and lateral funiculus at Th₈. In this case there was, in the ventromedial area studied, a complete absence of labeled cells at rostral levels I to III. From here down, through level VI, a sparse labeling were found ipsilaterally, and only very few 5HT cells were seen to be labeled in the ventral RM and among the fibres of the corticospinal tract. At the levels of the inferior olive some labeled 5HT cells were seen in RP and RO, but only one or two in the arcuate region. All of the labeled 5HT cells were found ipsilaterally as were also the majority of the few labeled non-5HT cells which were distributed within RO and just dorsal to the inferior olive.

The more medial injection at Th₈ depicted in Fig. 9b (rat 01-13) resulted in less labeling in RO, but exhibited a slightly higher number of labeled 5HT cells in RP as compared to the more laterally placed injection in rat 01-04 (illustrated in Fig. 9a). It is notable that in this case there was no clear ipsilateral dominance of labeled cells, while in all other respects the labeling was very similar to that observed after the more lateral injection.

As an example of the retrograde labeling obtained after injections restricted to the ventral and intermediate grey matter at the level of the IML the labeling in rat 05-14 is presented in Fig. 10a. In this animal labeled 5HT cells were seen in RP, RO, RV, RGC α , PGCL and the arcuate nucleus while no such cells were seen in RM or RMr. Occasional labeled non-5HT cells were seen in the latter nuclei.

Injections into the lumbosacral cord below the level of the IML

Throughout these levels tracer injections retrogradely labeled cells in the ventromedial caudal brainstem in a fashion very similar to that obtained after more rostral injections. In most areas, except the ipsilateral NIH, there was an easily discernible decrease in the overall number of labeled cells, most notable in the arcuate nucleus and in the RP, RO and RV.

To provide a survey of the retrograde labeling after lower lumbar injections, Fig. 10b illustrates the labeling recorded in rat 09-20 after a large injection which completely filled the hemichord at L₆. The labeling seen thus represents cells that projects to or through this segment.

At the most rostral levels (I-III) a number of labeled cells were seen bilaterally in RMr and the surrounding reticular formation. Of these only a minority (about 20%) were serotonergic. At the level of the facial nucleus gradually more cells were labeled in the RM, RGC and PGCL in which nuclei a minority of labeled 5HT-containing cells were found among the labeled non-5HT cells (Fig. 18). Many labeled non-5HT cells were also seen more dorsally, in RGC. There was some ipsilateral dominance of labeled cells, both 5HT and non-5HT-containing. At the levels of the RP and RO several labeled 5HT cells were encountered within RP and RO together with a slightly lower number of labeled non-5HT cells. No cells were seen in the contralateral NIH but in the ipsilateral nucleus the number of labeled cells was similar to that seen after the large cervical injection in rat 08-12 (Fig. 4a). In RP, RO and RV, and especially in the arcuate nucleus there were markedly fewer labeled cells compared to what was seen after more rostral injections (see Table I and Section VI, below).

Fig. 11a shows the result of an intermediate injection at L₅ in rat 11-13 which was confined to the grey matter. In this animal retrograde labeling was seen already at the most rostral levels where a varying minority of the TB labeled cells were 5HT-containing. Going caudally there was an increase in the number of labeled cells both 5HT and non-5HT ones, and the ipsilateral dominance was more pronounced. No labeled cells were found in the rostral parts of PGCL. At the level of the inferior olive about an equal number of labeled 5HT- and non-5HT-containing cells were seen in RO; in the RP a smaller number of exclusively 5HT-containing cells were labeled. At these levels also a fair number of labeled 5HT cells were found in PGCL and NIH with only a slight ipsilateral dominance. In the RCG α and RV labeled non-5HT cells were mainly distributed ipsilaterally.

Injections at sacral levels

Due to the small size of the sacral spinal cord and the difficulty involved in separating the spinal roots from the cord during the injection, only a small number of successful injections were obtained from these levels. After such injections there was a pronounced reduction in number of labeled cells in the brainstem as compared to what was seen after more rostral injections.

This decrease in retrograde labeling was seen throughout all but one of the nuclear areas studied, although it was most striking in RMr, RGC and caudal RP; in the arcuate nucleus there was a complete absence of labeled cells after injections below S₁. The NIH stands out as an exception; in this nucleus a good number of cells (virtually all 5HT-containing) could be retrogradely labeled even after injections at or below S₄.

In rat 81-11 (Fig 11b) the injection was centered at S₁ and covered part of the lateral funiculus and the lateral part of the grey matter. It thus included the area of preganglionic parasympathetic cell bodies. In this animal there was a general decrease in retrograde labeling, particularly in RGC and RP, but still some labeling was seen in RMr and the arcuate nucleus.

As a comparison rat 02-02 (Fig. 12a) received a more medially placed injection centered at S₃. It left the dorsal part of the lateral funiculus intact while filling most of the grey matter and most of the ventral funiculus. Also in this case there was a decrease in labeling in RMr and no labeled arcuate cells.

The last injection to be accounted for was obtained in rat 04-20 and is illustrated in Fig. 12b. This is the most caudal injection in the material having its largest extent at S₄ and upper coccygeal levels. Due to the small size of the cord at this level this small injection covered more than half of the cord. In this animal there was a very obvious decrease in the number of labeled cells in the brain-stem both with respect to 5HT and non-5HT-containing neurons. In RMr labeled cells were found only in the caudal part of the nucleus and only few scattered neurons could be seen in RPC or RGC. Most of the retrogradely labeled 5HT cells were found within RM, PGCL and rostral RO and RP. At the level of the inferior olive there was a rapid decrease in the number of labeled cells with only few labeled 5HT cells in RP, RO and NIH. No labeled cells were seen in the arcuate region while the "bridge" between the medial and lateral 5HT cell groups was clearly marked at the rostral levels of the inferior olive.

VI. Quantitative data from large injections at cervical, lumbar and sacral levels

In rats 08-12, 09-20, and 04-20, which received large hemichord injections at C₅, L₆ and S₄, respectively, retrogradely labeled 5HT and non-5HT cells were counted throughout the regions delineated in Fig. 1a. These results are summarized in Table I, which also lists the absolute number of 5HT-containing cells in the same regions in one of the animals. In the animal with the large cervical injection, the relative number of labeled 5HT cells was highest in RMr, RGC α and RV in which more than 50% of the total number of TB-labeled cells in the areas were labeled after the cervical hemichord injection, thus indicating that at least some 5HT cells in these areas send collaterals to (or through) both sides of the cord, and that probably all of them projects to the spinal cord. In PGCL, RP, NIH and arcuate nucleus 35-45% of the 5HT neurons on both sides were retrogradely labeled, possibly indicating that most of these serotonergic cells projects to the spinal cord, and do so either ipsi- or contralaterally. Considering the 5HT cells in RPC, RM and RO less than a third of these were found to be retrogradely labeled after this cervical hemichord injection, indicating that a substantial portion of the 5HT-neurons in these nuclei do not project to or through C₅.



Figure 13. Retrograde labeling seen in association with mesencephalic 5HT cell groups after a cervical hemichord injection (rat 08-12).

At each level, (a) being the most rostral, each symbol indicates one labeled serotonergic (filled stars) or non-serotonergic cell (unfilled stars) in a corresponding 15 μ m section.

Abbreviations. Aq, cerebral aqueduct; BP, brachium pontis; CG, crus cerebri; CG, central grey; CS, nucleus centralis superior; DR, nucleus raphe dorsalis; DTGD, dorsal tegmental decussation; FLM, medial longitudinal fasciculus; IPC, central part of interpeduncular nucleus; IPIP, inner part of posterior subnucleus of the interpeduncular nucleus; IL, lateral lemniscus; ML, medial lemniscus; PBC, parabigeminal nucleus; RTG, nucleus reticularis tegmenti pontis; SCP, superior cerebellar peduncle; VLL, ventral nucleus of the lateral lemniscus; VTG, ventral tegmental nucleus; III, oculomotor nucleus.

Looking at the total number of 5HT cells labeled in these regions after the hemichord injection at C₅ it can be seen that they constituted about 40% (2053 out of 5009) of the total 5HT cell population and that almost a third of them was found contralateral to the injection. This general arrangement remains very much the same also when studying separately the cell groups which preferentially projects to dorsal cord areas (i.e. RMr+RPC+RM+RGC α +PGCL) or to more ventral aspects (i.e. RP+RO+RV+NIH).

Especially in the arcuate cell group, but also in RP and RO, most of the labeled cells were 5HT-containing, while in the NIH there was as many 5HT as non-5HT-neurons among the labeled cells. In the other areas the TB-labeled non-5HT cells were clearly in majority, often outnumbering the labeled serotonergic neurons by a factor of two or more.

Both 5HT and non-5HT cells with retrograde labeling were most numerous ipsilaterally, with the exception of RM and RP where the labeled non-5HT cells seemed evenly distributed on both sides.

Comparing the values given for injections at C₅ and L₆ the decrease in number of cells labeled from the lower level was most marked in nucleus arcuatus (-70%), less prominent in RP, RO, RV and RGC and relatively small in RMr, RM, RGC α and PGCL and almost absent in NIH.

The decrease in number of labeled 5HT cells were somewhat more marked in areas projecting ventrally (RP+RO+RV+NIH) than in regions which preferentially projects to the dorsal cord (RMr+RPC+RM+RGC α +PGCL). Overall the decrease in number of labeled 5HT cells after the lumbar injection is much smaller than the corresponding decrease in spinal cord tissue present below (and at) the level of the injection as compared to the situation after the cervical injection.

After injection at S₄ the decrease in number of labeled cells was readily apparent throughout all the nuclear regions, still, however, a considerable number of TB-containing neurons were seen, especially in RM, RGC and RP.

VII. Retrograde labeling in association with mesencephalic 5HT cell groups

In the mesencephalon labeled 5HT cells only were seen after injections into the cervical cord. The pattern of labeling seen in this region was quite constant irrespective of the appearance of the cervical injection. We will therefore here limit our description to the labeling seen after a cervical hemichord injection (rat 08-12). In this animal TB-labeled cells were found close to or within the DR throughout its length (Fig. 13). Both 5HT and non-5HT-containing cells were most numerous on the side ipsilateral to the injection. Of the TB-labeled 5HT cells most were located ventrolaterally to the DR but some were clearly within the confines of the DR while only single labeled cells were seen close to the nucleus centralis superior or the adjacent B9 group. A total number of 62 5HT-containing cells in this area were found to be retrogradely labeled in this experiment. After injections below cervical levels a few labeled cells could still be seen in these mesencephalic regions, these were, however, after such lower injections never observed to contain 5HT. Such labeled non-serotonergic cells could be seen after injections throughout the thoracic cord but was not observed after injections at or below midlumbar levels.

Because of practical difficulties it was not possible to deduce if the 5HT projection from the mesencephalon preferentially terminates in dorsal or ventral regions of the cervical cord. It was noted though

that, while the cervical injections need not involve DLF in order to label 5HT cells in mesencephalon, the non-5HT cells close to the serotonergic cell groups that could be labeled from thoracic and upper lumbar injections apparently project through dorsal cord areas.

Discussion

Comments on methodology

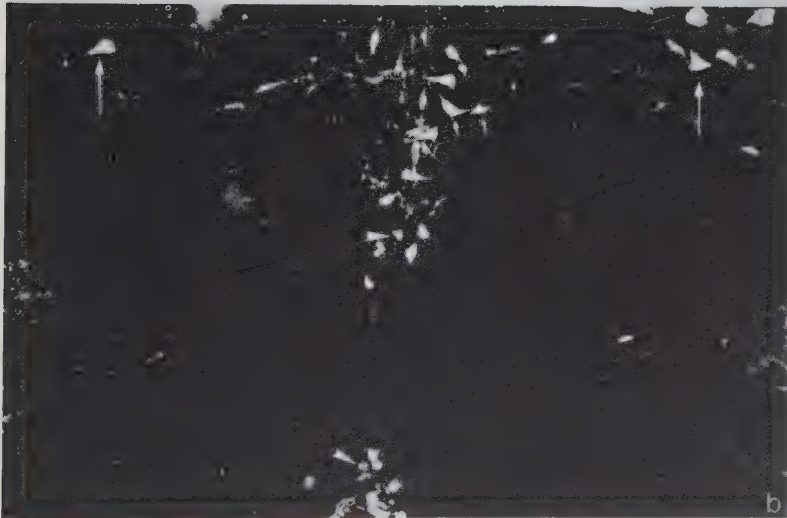
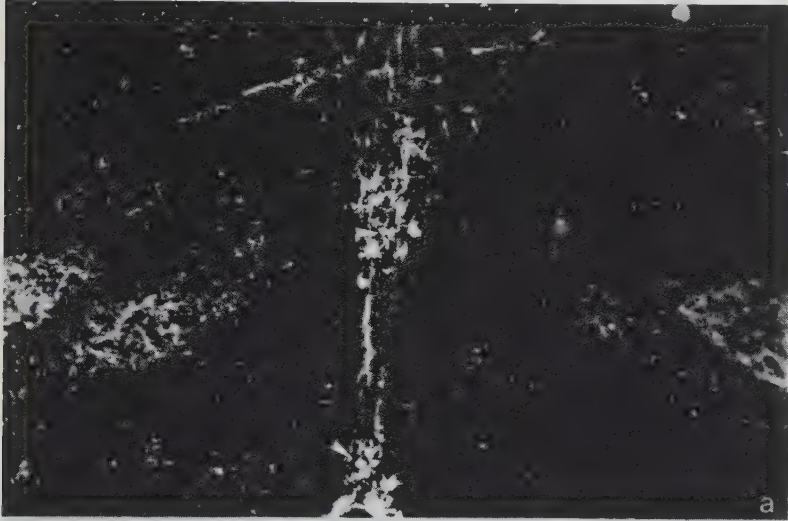
In the combined TB-ALFA procedure the distinction between labeled and non-labeled neurons is usually easy to make, provided that the survival time is correctly chosen. In specimens with strong 5HT fluorescence some individual 5HT-containing cells did only show very small changes in emission colour or morphology as the excitation light was changed between 365 and 435 nm. Such 5HT cells, in which tracer might have been present but could not be detected with certainty, constituted in all cases less than 10% of the number of TB-labeled 5HT cells in any given region and were always classified as unlabeled. The existence of a small population of 5HT cells, which contained amounts of the retrogradely transported tracer below the level of detection is suggested by the fact that occasional TB-labeled non-5HT neurons exhibited a tracer fluorescence of such weak intensity that it most likely would have been unnoticed if it had been contained within a neuron displaying 5HT fluorescence.

As concerns the injection sites it must be kept in mind that the extent of the peripheral zone is subjectively determined and, furthermore, that the degree of uptake from this zone is probably not uniform. That the TB suspension tends to diffuse only to a limited extent is usually advantageous for making small injections but becomes less of an asset when trying to completely fill the hemichord as in the injection shown in Fig. 4a. Thus, although we believe that these large injections provide much useful information, they call for cautious interpretation especially as concerns absolute numbers of labeled cells, since individual fibres may possibly have passed through the injected area without taking up the tracer. It should also be remembered that there are no really distinct borders between the different nuclear areas delineated in this report and that therefore the referral of individual cells to a certain region is sometimes a very subjective process.

Serotonergic and non-serotonergic components in the raphe-spinal system

The present study clearly demonstrates that from most brainstem nuclei that gives rise to spinal 5HT projections there is also an, often larger non-5HT projection to the cord. As mentioned above, the existence of spinal non-5HT projections from these areas has been suggested by several lines of evidence, both electrophysiological^{71,72} and anatomical⁷³, and functional studies on the analgesic effects mediated via the raphe-spinal pathways have indicated that both serotonergic⁷⁶ and non-serotonergic^{38,43} mechanisms are involved (see^{10,29} for reviews). Direct anatomical evidence of such a non-serotonergic spinal projection was first provided by Bowker et al.¹⁵ who have since also partly characterized the non-serotonergic component¹⁷. In comparison with the quantitative data from this latter group¹⁶, which showed the non-serotonergic component to amount to about 10% of the spinal projecting neurons in the area of the B1-B3 group, our results point to a much larger non-serotonergic component especially in the more rostral regions (PGCL, RGC α , RM, RMr). This discrepancy can in our view, only partly be explained by the fact that we have counted

Figure 14. (a) Distribution of serotonergic cells in RO (dorsally) and RP (ventrally) approximately at level VII. In (b) the retrograde labeling is visualized at 365 nm excitation. Several retrogradely labeled serotonergic neurons are seen especially ipsilaterally, (right) in RP and RO, some of such cells are also seen just on the border to RV (small arrows). Long arrows point to some of the labeled non-serotonergic neurons in RV. Arrowheads indicate some unlabeled serotonergic cells in RP. Note the heavy monoaminergic innervation of RP and RO, the densely innervated areas laterally demarcate the principal part of the inferior olive. X100.



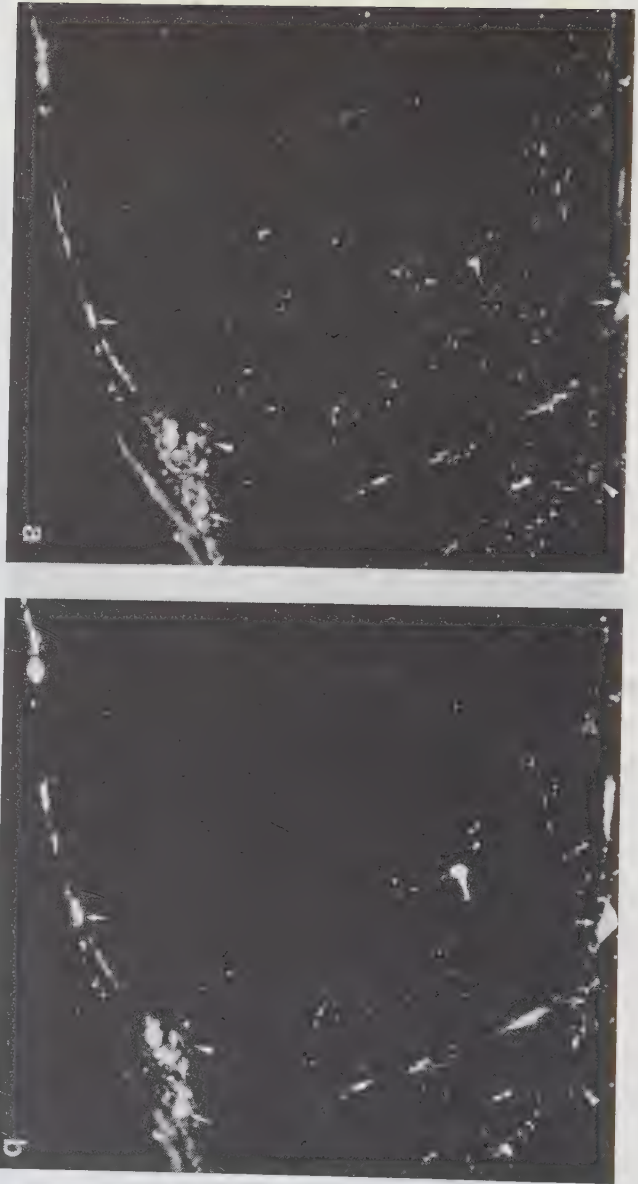


Figure 15. Retrograde labeling in the arcuate cell group and PGCL after a tracer injection into the lateral funiculus at midcervical level. In (a) several small serotonergic arcuate cells can be seen just ventral and ventrolateral to the pyramidal tract. Some larger 5HT cells are also seen more dorsally in the PGCL. Excitation at 365 nm (b) reveals that many of the serotonergic cells are retrogradely labeled. Small arrows point to some of the retrogradely labeled serotonergic cells, arrowheads indicate serotonergic neurons that have not taken up the tracer. X154.

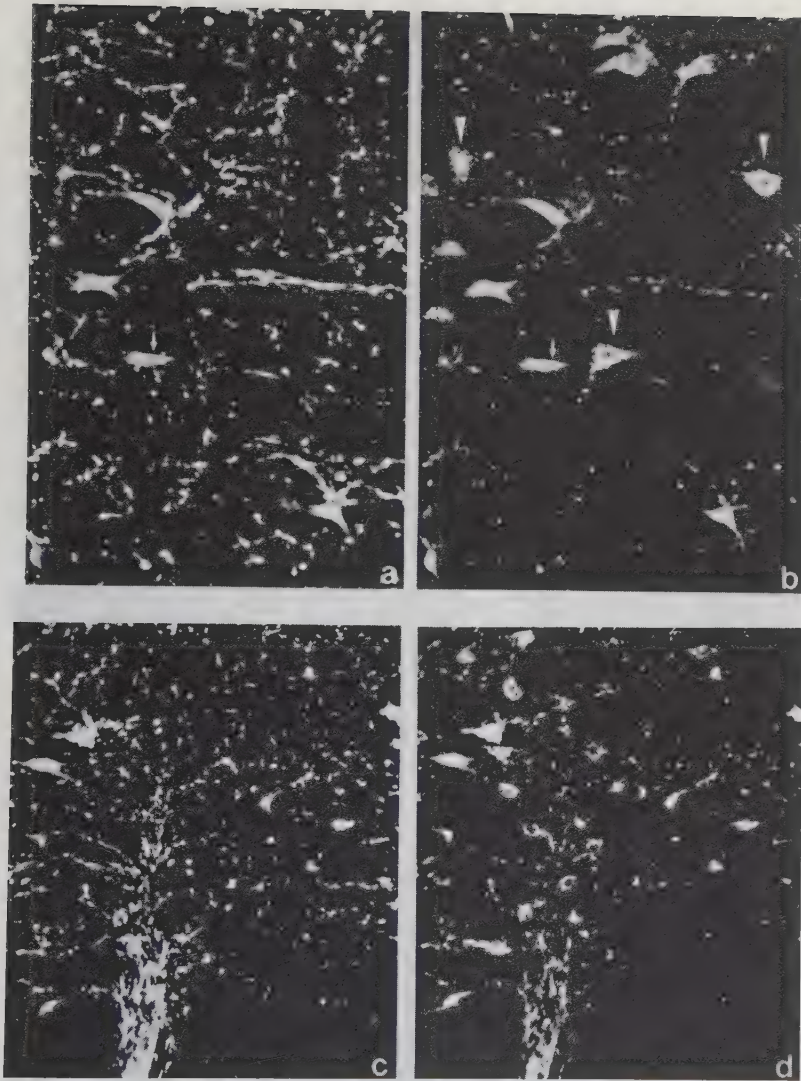


Figure 16. These micrographs illustrate the large number of retrogradely labeled cells seen after an injection into the lateral funiculus at midcervical level. In (a) excitation at 435 nm shows serotonergic cell-bodies and monoaminergic fibers and terminals in RGC α . In (b) the same field of view is excited at 365 nm and thus visualizes labeled serotonergic cells (small arrow) as well as labeled non-serotonergic cells (arrowheads). X220. In (c) monoaminefluorescence is visualized in the region of RM, the dark areas in the bottom of the micrograph correspond to part of the pyramidal tracts. With 365 nm excitation (d) several labeled nonserotonergic cells are added to the picture. X112.

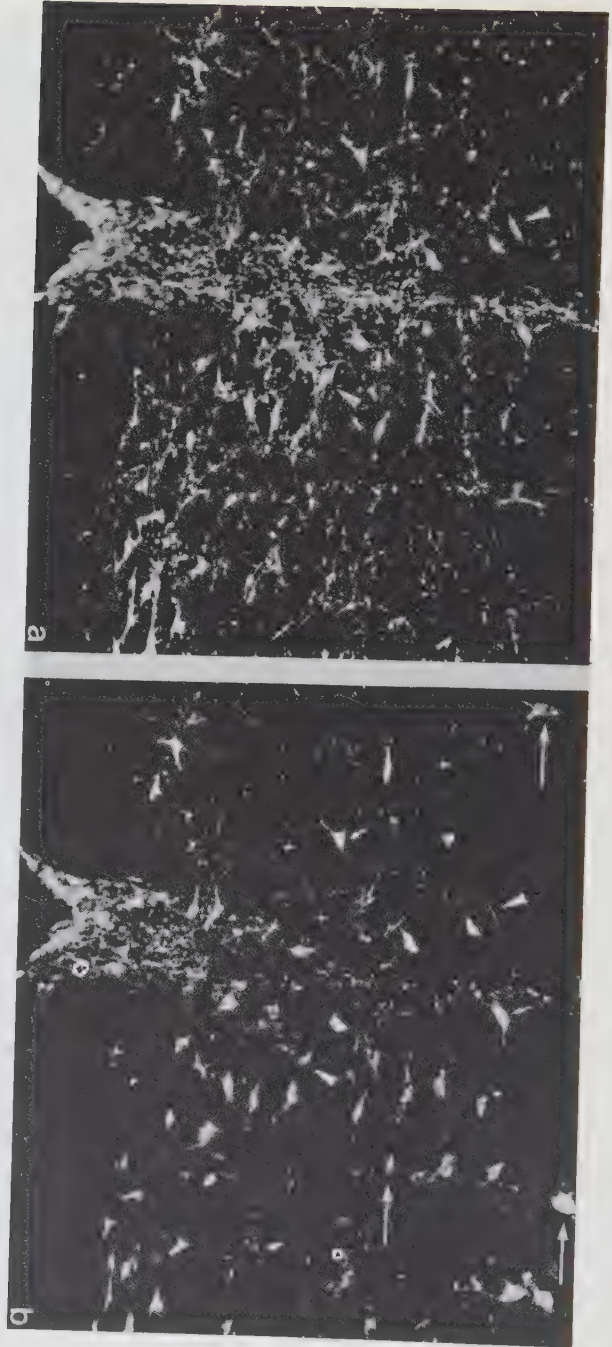


Figure 17. Retrograde labeling in RM, RGC α and RGC close to level IV after a tracer injection into the dorsal part of the spinal cord at mid-thoracic level. At 435 nm excitation (a) several serotonergic cells and a fairly dense monoaminergic innervation are seen dorsal to the pyramidal tracts which are essentially devoid of fluorescence. In (b) the filter setting for TB visualizes retrogradely labeled 5HT cells (some marked with small arrows), unlabeled 5HT cells (some marked with arrowheads) and also labeled non-5HT cells (some marked with long arrows). X84.

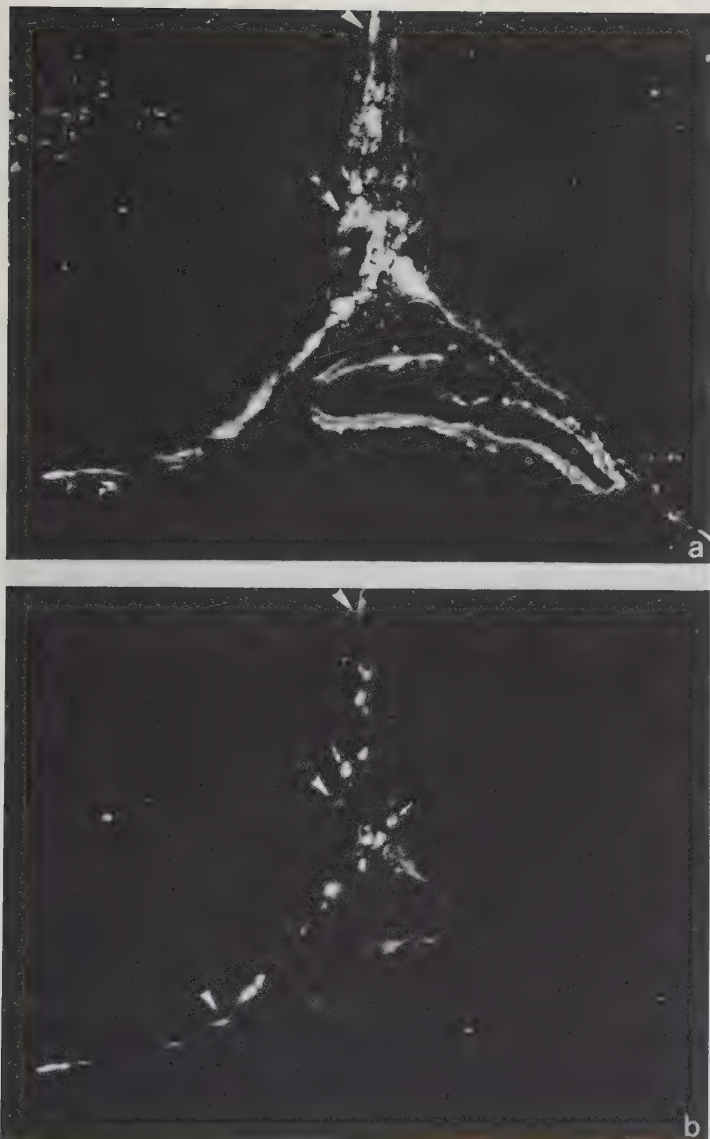


Figure 18. Retrograde labeling in RP and in cells belonging to the arcuate cell group after a thoracic injection which covered most of the lateral funiculus. The two pyramids are seen overlying a bloodvessel at approximately level VIII. In (a) only serotonergic cells are visualized while at 365 nm (b) some of these are seen to be retrogradely labeled (some marked with small arrows). Arrowheads points to unlabeled serotonergic neurons. 120X.

labeled neurons in a larger area since in most nuclear regions the labeled 5HT and non-5HT neurons were closely situated or actually intermingled. It is noteworthy that in the abstract by Johannesen et al.³⁹ very few 5HT neurons in these regions were reported to be labeled by tracer deposition in the DLF while a large number of labeled non-5HT cells were seen. Thus, the results presented in this paper are in partial (dis-)agreement with both studies. The fact that the number number of TB labeled 5HT cells found by us was very similar to that of Bowker et al.¹⁶, but that we observed a markedly higher total number of retrogradely labeled neurons could possibly indicate that TB is more efficiently transported (or visualized) than horse-radish-peroxidase⁵⁹.

Collateralization of the raphe-spinal systems

In RMR, RM, RGC α , PGCL and NIH we observed only a small decrease in the number of labeled (5HT and non-5HT) cells after injections at L₆, as compared to C₅, but we also noted that many cells in these regions could be labeled after injections that were confined to the grey matter above the L₆ level. This points to a mode of organization where many neurons send axons that traverse the length of the whole cord while fairly frequently distributing collaterals to the grey matter. Such collateralization of raphespinal neurons has also been implicated in earlier electrophysiological⁵⁵ and anatomical studies^{37,49}. By using two different retrograde tracers simultaneously, Huisman et al.³⁷ and Martin et al.⁴⁹ were able to demonstrate spinal collateralization from single (chemically unidentified) neurons in RM or RO. This is thus compatible with our findings which, however, indicate that this phenomenon is a less common feature of neurons in RP, RO, RV and the arcuate nucleus than in RM.

The large proportion (>50%) of 5HT neurons that could be labeled in RM, RGC α , PGCL and RV after cervical hemichord injections indicates that, besides the fact that probably all 5-HT neurons in these areas project to the spinal cord, a significant fraction of these cells sends collaterals to or through both sides of the cord. Comparing the results obtained from injections confined to either grey or white matter it seems, however, that most of the 5HT axons descend through ipsilateral funicular trajectories but that they to some degree cross at the segmental level. The descending non-5HT systems, on the other hand, show more of a bilateral distribution also as concerns their funicular trajectories.

From the results presented in Table I it seems evident that a significant proportion of the 5HT cell population in RM, RP, RV and NIH does not project to or through C₅ but must have other target areas. From our findings that the 5HT neurons in RM show a relatively limited degree of collateralization and that they mainly project to sensory areas of the cord, it seems likely that many of the RM neurons have projections restricted to more rostral levels of the dorsal cord or to the spinal trigeminal nucleus in the brainstem¹¹. Other 5HT neurons in this area may, however, project more rostrally through the medial forebrain bundle^{63,64}. By the same line of argument individual 5HT neurons in the RP and RO are likely to project rostrally to somatic motoneuron nuclei of the cranial nerves, which have been shown to receive a dense serotonergic innervation^{81,61,65}.

The spinal projection from the arcuate cell group is remarkable in many respects. This peculiar cell group is made up of many small rounded neurons which are situated so close to the pia of the ventral surface of the brainstem that Steinbusch⁶² has considered at least some of them to be extracerebral. Our results are somewhat variable

TABLE I

	RM+	RPC	RM	RGC	RGC α	PGCL	RM+RPC+RM+ RGC α +PGCL
5HT cells							
total	183	243	478	0	471	519	1894
ipsi.	(92)	(122)	(239)	(0)	(236)	(260)	(947)
C ₅ (rat 08-12)							
ipsi.	85:126 (92%)	57:114 (47%)	109:258 (46%)	0:721 (0%)	147:510 (62%)	147:225 (57%)	545:1155 (57%)
contra.	36: 72 (39%)	12: 34 (10%)	45:270 (19%)	0:261 (0%)	99:351 (42%)	45:156 (17%)	237: 883 (25%)
L ₆ (rat 09-20)							
ipsi.	69: 92 (75%)	15: 99 (13%)	70:140 (30%)	0:370 (0%)	112:433 (47%)	90:145 (35%)	356: 909 (37%)
contra.	14: 84 (15%)	12: 40 (10%)	20:120 (8%)	0:180 (0%)	40:300 (17%)	27: 75 (10%)	113: 619 (12%)
S ₄ (rat 04-20)							
ipsi.	9: 39 (10%)	0: 45 (0%)	40:120 (17%)	0: 15 (0%)	10:195 (4%)	36: 30 (14%)	95: 424 (10%)
contra.	0: 20 (0%)	0: 15 (0%)	10: 75 (4%)	0: 0 (0%)	0: 60 (0%)	30: 0 (11%)	40: 200 (5%)

Table I. Number of retrogradely labeled cells after hemicord injections at cervical lumbar and sacral levels.

The uppermost row gives the total number of 5HT cells in the different regions in one animal. In brackets below is half the number signifying the cells on one side. The numbers of retrogradely labeled cells are then given for the areas ipsilateral (ipsi.) and contralateral (contra.) to the injection site. In each group of three numbers the first gives the number of retrogradely labeled 5HT cells, the second number (after the colon) is the number of labeled non-serotonergic cells. In brackets is given the percentage of labeled 5HT cells on that side.

(continuing next page)

TABLE I (cont.)

	Arc	RP	RO	RV	RP+RO+ RV+NIH	NIH	Total, all regions
SHP cells	total	1014	870	864	235	2101	132
	ipsi.	(507)	(435)	(432)	(118)	(1050)	(67)
							(2504)
C ₅ (rat 08-12)	ipsi.	321:69 (63%)	252:77 (58%)	195:66 (45%)	84:580 (71%)	577:1372 (55%)	46:36 (67%)
	contra.	129: 3 (25%)	98:60 (22%)	81:39 (19%)	61:278 (52%)	244: 392 (23%)	4:15 (6%)
							1443:2782 (58%)
L ₆ (rat 09-20)	ipsi.	45:51 (9%)	135:30 (31%)	60:72 (14%)	21:312 (18%)	261: 444 (25%)	45:30 (67%)
	contra.	26:18 (5%)	45:42 (8%)	45:60 (10%)	4:150 (3%)	94: 252 (9%)	0: 0 (0%)
							662:1774 (26%)
S ₄ (rat 04-20)	ipsi.	0: 0 (0%)	75:10 (17%)	0:21 (0%)	0: 50 (0%)	100: 81 (17%)	25: 0 (37%)
	contra.	0: 0 (0%)	0:10 (0%)	10: 0 (2%)	0: 15 (0%)	10: 25 (2%)	0: 0 (0%)
							195: 699 (8%)
							50: 185 (2%)

with respect to a non-serotonergic component of the population of arcuate neurons. Whether this reflects a fluctuation in the 5HT content of these cells or the presence of a pure non-serotonergic component cannot be established at this point. It may be of importance, however, that these neurons differ from the more dorsally located 5HT cells by only rarely containing substance P⁴⁰. Although our results indicate that the arcuate cell group provides the quantitatively dominating 5HT-projection to the IML it should be noted that our results also confirm the presence of serotonergic projections to this area from RMR, PGCL and to a lesser degree also from RP and RO, as previously reported^{8,36,45,46}.

Delineation and functional aspects of the dorsal, intermediate and ventral 5HT pathways

While our study has not directly visualized the funicular trajectories of the descending 5HT pathways we feel that it is possible to discern three anatomically and functionally distinct subsystems¹⁴, as illustrated schematically in Fig. 18.

(1) The dorsal pathway consists of axons in the DLF which originate mainly in RMR, RM, RGC α and PGCL and which project to the dorsal horn and the intermediate zone. This system comprises both serotonergic and non-serotonergic components, both of which mainly descends ipsilaterally but to some extent cross at the segmental level of termination.

(2) The intermediate pathway consists of axons that project to the intermediate zone, especially to the region of preganglionic sympathetic and parasympathetic autonomic neurons. These axons originate mainly from serotonergic neurons in the ipsilateral arcuate cell group but also from cell bodies in RP and RO.

(3) The ventral pathway projects through the ventral and ventrolateral funiculi to the area of somatic motoneurons in the ventral horn. The serotonergic component originates largely in RP, RO and to some degree in NIH, while non-serotonergic axons also emanates from RV and from more rostral areas, i.e. the caudal parts of RM and RGC α .

The delineation of a separate dorsal pathway with axons descending in the DLF and originating mainly in RMR, RM, RGC α , PGCL and partly in RV has been amply documented in several studies using both anterograde and retrograde tracing techniques in various species^{8,9,35,51,67,77}. Besides some termination in the intermediate zone⁸ most of these axons appears to be distributed to regions of the dorsal horn which contains spino-thalamic tract neurons onto which activity in this pathway conveys a powerful inhibition¹⁰. As clearly demonstrated in this report a large part of this dorsal pathway consists of non-serotonergic projections, a finding which may explain why brain stem descending inhibition and analgesia has been found to be partly independent on serotonergic mechanisms in the dorsal horn^{43,38}. From other studies, it seems, however, that the 5HT component of the dorsal pathway is clearly implicated in descending spinal analgesic mechanisms⁷⁶.

While the technique used in this study distinguishes between a serotonergic and a non-serotonergic component of the dorsal pathway, it is important to realize that these two components are not in themselves homogenous with respect to transmitter content⁷⁰. Thus, as mentioned in the Introduction, at least some of the 5HT neurons that project along the dorsal pathway probably also contain TRH and substance P, either alone or together, while others contain enkephalin^{17,40}. These substances (as well as cholecystokinin⁴⁸) may also be present in the non-serotonergic component of this pathway although the transmitter content of this part of the system is largely unknown.

DESCENDING 5HT-SYSTEMS

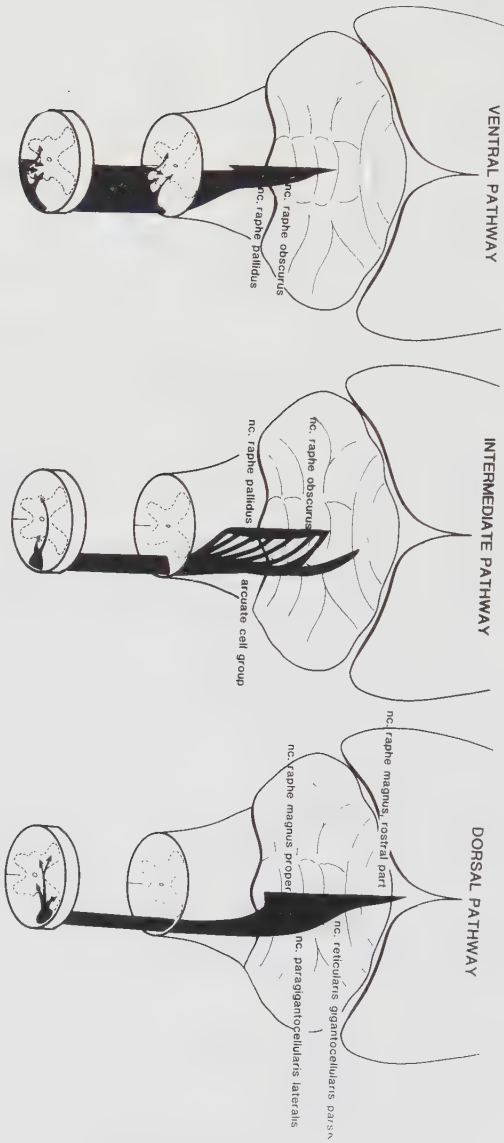


Figure 19. Schematic drawings illustrating the anatomical organization of the descending serotonergic spinal projections.

There is substantial evidence that the dorsal pathway is tightly coupled to opiate systems. Not only are many of these neurons excited (through disinhibition) by opiates³¹ but also the inhibitory effect at the spinal level does appear to be dependent on a local opiate link^{32,78}. The exact mechanism whereby the system exerts an inhibitory influence on sensory mechanisms is however not known and may, in the light of our present knowledge of the different transmitter combinations involved, be quite complex²⁷.

The intermediate pathway seems to be mostly serotonergic and distributes its terminals mainly to areas of preganglionic autonomic motoneurons in the lumbar and sacral spinal cord. While this projection may be partly inhibitory in birds^{19,20} there is now much evidence favoring the view that this system, at least with respect to its serotonergic component, provides an excitatory input onto the cells of the IML in mammals^{24,36,52}. That this pathway originates mainly in the arcuate region is evidenced by the fact that single 5HT cells in this area can be labeled after small IML injections and by our quantitative data which indicates that most of these neurons project to thoracolumbar levels. A spinal projection from this area was first documented by Ross et al.⁵⁸ who, by considering its overlap with a ventral chemosensitive area³⁰ speculated on the possibility that these neurons act as chemosensors relaying chemical influences in the cerebrospinal fluid onto the autonomic nervous system. Besides the arcuate nucleus this pathway also contains fibres from RP and RO projecting to the IML⁴⁶.

The ventral pathway has a large non-serotonergic component with many of its cell bodies of origin located dorsal to the areas rich in 5HT-containing cell bodies. The serotonergic component originates in RP and RO, and descends in the ventral white matter. Many of these fibres seem to terminate in close contact with motoneurons in the ventral horn^{26,41,42}. While the ventral pathway as a whole may mediate both excitatory and inhibitory influences onto spinal motoneurons⁵⁵ most recent data indicates that serotonin increases the excitability of anterior horn motoneurons^{4,78}.

Besides the inputs to RM and immediately surrounding regions which are gradually being worked out^{2,74}, little is known about the afferents to the more caudally situated regions, which give rise to the ventral and intermediate pathway. The finding that virtually all spinal-projecting 5HT cells are located in areas with a dense catecholamine innervation suggests, however, that noradrenergic (and possibly also dopaminergic) systems may exert indirect effects on spinal somatic and autonomic motor function as well as on sensory mechanisms^{33,43}.

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